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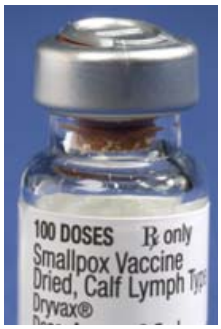
On the Cover

Eugène-Ernest Hillemacher
(1818–1887)
Edward Jenner Vaccinating a Boy (1884)
Oil on canvas (73.1 cm × 92.7 cm)

About the Cover p. 763

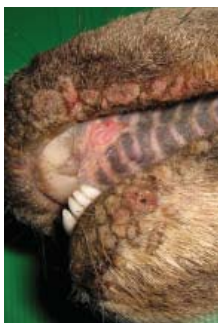
Synopsis

Research



p. 682

p. 685



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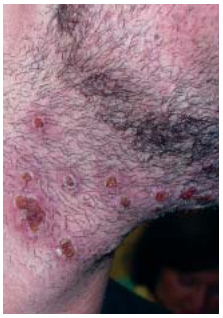
April 2011

Policy Reviews



p. 720

Dispatches

p. 759

Another Dimension

Letters

In Memoriam

About the Cover

Legionella longbeachae and Legionellosis

Harriet Whiley and Richard Bentham

Reported cases of legionellosis attributable to *Legionella longbeachae* infection have increased worldwide. In Australia and New Zealand, *L. longbeachae* has been a known cause of legionellosis since the late 1980s. All cases for which a source was confirmed were associated with potting mixes and composts. Unlike the situation with other *Legionella* spp., *L. longbeachae*–contaminated water systems in the built environment that cause disease have not been reported. Spatially and temporally linked outbreaks of legionellosis associated with this organism also have not been reported. Sporadic cases of disease seem to be limited to persons who have had direct contact with potting soil or compost. Long-distance travel of the organism resulting in infection has not been reported. These factors indicate emergence of an agent of legionellosis that differs in etiology from other species and possibly in route of disease transmission.

Legionella spp. were first identified as organisms of public health significance in 1976 and are now recognized as the causative agent of legionellosis. *L. pneumophila* was the species responsible for this initial disease outbreak and has remained the major cause of legionellosis (*1*,*2*). The clinical manifestations of legionellosis range from no symptoms to acute atypical pneumonia and multisystem disease (*2*). The term legionellosis refers collectively to the clinical syndromes resulting from *Legionella* spp. infection, i.e., Legionnaires' disease (a *Legionella* spp.–derived pneumonic infection) and Pontiac fever (an acute, self-limited febrile illness that has been linked serologically and by culture to *Legionella* spp.) (*1*,*2*). Community- and hospital-acquired legionelloses typically are associated with water systems in the built environment, such as cooling towers, spas, showers, and other warm water systems (*1*,*2*). Protozoa play a major role in the multiplication and dissemination of *Legionella* spp. in natural environments. The parasitism of amoebae and ciliates is well documented, and this parasitic capability is the basis of human disease through infection of human lung macrophages (*1*,*2*).

L. longbeachae was first isolated in 1980 from a patient with pneumonia in Long Beach, California, USA (*3*). A second serogroup of *L. longbeachae* was discovered during the same year (*4*). Neither of these reports suggested a recognized source of infection.

In Europe, *L. pneumophila* is responsible for 95% of cases of Legionnaires' disease. Of the remaining 5%, the most common causative agent is *L. longbeachae* (*5*). In Australia, New Zealand, and Japan, reported cases of *L. longbeachae* infection occur as often as cases of *L. pneumophila* infection (*6*–*8*). Within the past decade, the number of *L. longbeachae* reports has increased markedly across Europe and parts of Asia (*9*–*15*).

Potting Mixes

L. pneumophila is primarily aquatic and endemic to warm water in the built environment (e.g., cooling towers, shower heads, and water fountains) and in natural environments (e.g., rivers and lakes) (*1*,*2*). It is transmitted from the environment through inhalation of aerosol or aspiration of *Legionella* spp.–contaminated particles (*1*,*2*). *L. longbeachae* is rarely isolated from aquatic environments (*16*,*17*). The primary environmental reservoir of *L. longbeachae* remains unknown; however, the major source of human infection is considered to be commercial potting mixes and other decomposing materials, such as bark and sawdust (*5*,*8*,*18*,*19*). No reports of *L. longbeachae*

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infection from water systems in the built environment have been confirmed.

Recent analysis of the *L. longbeachae* genome has demonstrated that it is highly adapted to the soil environment. The genome encodes for a range of proteins that might assist in the invasion and degradation of plant material (*20*). These enzyme systems are not present in *L. pneumophila*. This work supports the hypothesis of a possible environmental association with certain plant species (*8*,*18*).

The link between potting mix and legionellosis was established in 1989 when a cluster of *L. longbeachae* infections was detected in South Australia. Investigations identified commercial potting mixes as the source of disease (*18*). Since then, *L. longbeachae* commonly has been isolated from fresh potting mixes and some of its components but less commonly from natural soils, which suggests that the composting process may be a catalyst for growth. The heat and high moisture content during composting may allow for multiplication of several *Legionella* species to detectable levels (*18*). The route of transmission of *L. longbeachae* from contaminated environmental samples remains unknown (*7*,*18*).

In 1990, a study determining the incidence of *Legionella* spp. in potting mix found that more than two thirds (33/45) of Australian potting mixes and none (0/19) of European potting mixes tested positive for *Legionella* spp. (*18*). The authors postulated that the discrepancy between incidence of *L. longbeachae* infection in Australia and the rest of the world, particularly Europe, was attributable to the content of commercial potting mix. In Australia, potting mix is made mostly from composted pine waste products, such as sawdust and hammer-milled bark. In Europe, peat is the main component of potting mix (*16*,*18*). In 2001, a similar study in Japan found that 2 of 24 commercial potting mixes contained *L. longbeachae*. The main component of Japanese potting mix is composted wood products, particularly composted oak. The Japanese study also found that an amoebic enrichment of the potting mixes resulted in 9 of 24 potting mixes testing positive for *L. longbeachae*. This finding demonstrated that *L. longbeachae* can parasitize soil protozoa and that it was present in potting mixes but at numbers lower than the limit detected by using culture (*8*). Genomic analysis subsequently confirmed this parasitic capability (*20*). In 2008, testing for *Legionella* spp. was conducted on 46 commercial potting mixes in Switzerland. Two of 46 were culture positive for *L. longbeachae* and almost half (21/46) for *Legionella* spp. Most (41/46) of the potting mixes tested positive by quantitative PCR for *Legionella* spp. Two thirds of these potting mixes contained peat as the base component. This result contradicted previous studies on European potting mixes but supported the emerging trend of increasing numbers of reported *L. longbeachae* cases (*12*,*18*).

Detection

Detection of *Legionella* spp. by culture techniques is insensitive. Overgrowth of culture media with competing flora is a major problem (*1*,*2*). This problem is heightened for detection of *L. longbeachae* in potting mixes. Potting mixes have a high microbial load and contain spore-forming bacteria and fungi associated with composting. As a result, heat pretreatment of potting mixes tends to stimulate germination of spores and rapid overgrowth of the agar medium, rather than reduce competing flora. Acid pretreatment is the preferred option (*18*). The variable nature, pH, buffering capacity, and humic content of commercial compost and potting mixes means that the duration of acid pretreatment is best tailored to the individual sample rather than being generically applied (*12*,*18*).

Molecular methods (quantitative PCR) have been used recently to quantitatively detect *Legionella* spp. in potting mixes when culture methods gave negative results (*11*). Improved but nonquantifiable detection in potting soils also have been reported after amoebic enrichment of soil samples (*8*).

Disease Prevalence

Clinical presentations of *L. longbeachae* infections are similar to those of other legionelloses (*21*). Risk factors for infection in common with other *Legionella* infections are smoking, preexisting medical conditions, and immunosuppression. Gardening activities and use of potting mixes are risk factors that are so far unique to *L. longbeachae* infection (*7*). The disease predominantly affects persons <50 years of age, and reports suggest the median age for infection is slightly higher for *L. longbeachae* than for *L. pneumophila* (*2*,*7*,*16*,*21*). In addition, fewer deaths tend to be associated with *L. longbeachae* infection than with *L. pneumophila* (*21*). The virulence factors associated with *L. longbeachae* clearly differ from those of *L. pneumophila*, which may help explain the differences in disease prevalence and severity (*20*).

Recently, *L. longbeachae*–derived Legionnaires' disease has increased worldwide. In the Netherlands during 2000–2004, the first 5 reported cases of *L. longbeachae*–derived pneumonia were reported (*13*). Potting mix was associated with infection when analysis found a genotypically identical strain of *L. longbeachae* in the patient's sputum and in the potting mix. Two other patients of the 5 had indistinguishable genotypes, 1 of whom had visited the same gardening center as the index patient. Unfortunately, further analysis of the cluster was not possible because 3 of the patients died after hospital admission (*13*).

In Thailand, a population-based survey was conducted during 2003–2004 on 556 pneumonia patients >18 years of age who received chest radiographs and etiologic testing. This study found no positive cases of *L. pneumophila* and 20 (5%) cases of *L. longbeachae*. The global increase in infection rates is associated with soils and potting mixes This study did not identify an environmental source of infection (*10*). In 2004, a 25-year-old woman in Spain who had systemic lupus erythematosus died of community-acquired *L. longbeachae*–derived pneumonia (*14*).

During 2008–2009, Scotland recorded a cluster of Legionnaires' disease caused by *L. longbeachae*. Potting mix was associated with all 3 cases of infection. *L. longbeachae* isolates from patients and potting mix were genotyped by amplified fragment-length polymorphism. The genotypes isolated from the first 2 patients matched the genotypes from the associated potting mixes. No isolate was available from the third patient, but the genotype from the potting mix matched the genotype from the first patient. The first 2 patients had contact with the same brand of potting mix, which contained composted green waste (heat treated at 65°C for 5–10 days) and 30%–50% peat that had not been heat treated. The second patient also had contact with a second brand of potting mix that contained 75%–80% peat that had not been heat treated. The third patient had contact with compost made from expanded wood fiber, coir, and bark (*22*).

These reports contrast with previous reports of *L. longbeachae* in Europe. In 1999, the European Working Group on Legionella Infections reported only 2 cases of *L. longbeachae* from a total of 337 (<1%) reported *Legionella* spp. infections (*22*). In 2008, *L. longbeachae* was noted as the dominant species among non–*L. pneumophila* infections in Europe (*23*).

The number of reported *L. longbeachae* cases might not truly represent the total numbers because the infection in many patients might go undiagnosed. Standard routine diagnostic testing for pneumonia patients involves a legionellosis urine antigen test, which detects only *L. pneumophila* serogroup 1 (*22*). Also, many patients with Pontiac fever might not require hospitalization and might not be aware they have a *Legionella* spp. infection (*1*).

Survival in the Environment

The mechanisms that enable *Legionella* spp. to infect protozoa also enable opportunistic infection of the alveolar macrophages within human lungs. *Legionella* spp. infect and multiply within protozoan hosts in the absence of any other supporting nutrients (*2*). The relationship between *L. pneumophila* and a range of protozoan hosts has been documented in detail (*1*,*2*). The relationship between *L. longbeachae* and protozoan hosts is not as well understood.

Experimentally, both *L. pneumophila* and *L. longbeachae* infected the ciliate *Tetrahymena pyriformis*, although protozoan susceptibility to infection varied according to strain differences and available nutrients (*24*). In addition, although in situ *L. pneumophila* can infect and multiply within *Acanthamoeba castellanii*, *L. longbeachae* is unable to do so (*25*). Recently both *L. pneumophila* and *L. longbeachae* have been shown to colonize and persist within the intestinal tracts of *Caenorhabditis* nematodes in laboratory assays and soil environments. *Legionella* spp. replicated within the intestinal tract but did not invade surrounding tissue and were excreted as differentiated forms similar in structure to protozoan cysts. This study suggested that nematodes may serve as natural hosts for *Legionella* spp. and assist in their propagation throughout soil environments. The ability of *L. longbeachae* to infect protozoan and metazoan hosts allows for long-term contamination of environmental sites (*26*). The ability to survive protozoan cyst formation might also explain ability of *L. longbeachae* to endure the composting process and survive in desiccated potting mixes (*16*,*18*).

Disease Transmission

Spatially and temporally linked Legionnaires' disease outbreaks associated with *L. longbeachae* have never been confirmed. The first cluster of cases detected in South Australia was reported as seasonally but not geographically related (*27*). Seasonal clustering of cases during spring and autumn has been noted in Australia and overseas (*22*,*27*).

Cases of disease typically are sporadic and statistically associated with potting mix use and gardening activities (*28*,*29*). The route of disease transmission remains uncertain, although close proximity or direct contact with composts and potting mixes support hand-to-mouth, aspiration, or aerosolization routes of infection (*7*). No reports have been published that detail infection associated with long-distance travel of *L. longbeachae*, which contrasts markedly with the considerable distances traveled by other *Legionella* spp. during disease outbreaks (*2*).

A recent report detailed an outbreak of *L. longbeachae* infection in a commercial nursery (*28*). In this instance, Pontiac fever was the clinical presentation. Workers were in an enclosed facility without respiratory protection and with considerable potential for dust and aerosol generation. This is the first report of either Pontiac fever or a temporally and spatially confirmed outbreak of legionellosis associated with *L. longbeachae* (*28*).

Reported cases of infection in Asia, Europe, and the United States follow a similar pattern of sporadic disease linked to direct exposure to potting mix and compost (*9*,*10*,*13*,*22*,*29*). The rarity of outbreaks of disease and prerequisite for direct exposure suggest an alternative route of transmission of disease to other *Legionella* spp., and the

literature alludes to this information (*7*,*18*). Concentrations of the organism per gram of potting mix have been reported that are comparable to those associated with Legionnaires' disease per milliliter attributed to water systems (*1*,*2*,*12*). In addition, other disease-causing legionellae are present in potting mixes (*8*,*18*). In only 1 instance has potting mix been (inconclusively) implicated as a possible source of Legionnaires' disease from an organism other than *L. longbeachae* (*30*). Why potting mix is a source of infection from only this species remains a mystery.

Currently, no strategies are available to control or eliminate *Legionella* spp. in potting mixes. Awareness of health risks associated with handling compost and potting mixes protects against disease; the precise nature of this protective effect is unknown (*7*). In Australia, all bagged potting mixes and compost carry a health warning and recommendations for how to avoid infection. These recommendations include using a face mask, avoiding inhalation of dust and aerosols, and washing hands after using the material (*31*).

Conclusions

L. longbeachae infections have accounted for a major proportion of legionelloses in Australia and New Zealand since the late 1980s (*7*). Recently, the global incidence of reported *L. longbeachae* infections has increased (*9*,*23*,*23*,*28*). Factors explaining this emergence of infections are unknown but may be result in part from improved surveillance (*23*). In all reports, disease transmission is associated with soils, composts, and potting mixes rather than with water systems, with which other *Legionella* spp. infections are associated (*7*,*18.27*). The mechanism of *L. longbeachae* transmission remains unknown, but close association with contaminated material is a recurrent theme (*7*). Long-distance travel of the organism and subsequent infection has not been documented, which may suggest that disease is not transmitted through aerosol inhalation (*7*,*18*,*27*). The environmental reservoir for this *Legionella* species is yet to be identified, and association with a range of plant materials has been postulated (*7*,*18*,*20*). Isolation from peat-based potting mixes confounds this theory to some extent (*12*,*13*). Control strategies for this emerging disease are limited to published warnings on bagged products relating to handling and exposure (*7*,*22*,*31*).

Dr Whiley is a postgraduate student at Flinders University, Adelaide, South Australia, Australia. Her research focuses on the molecular detection of *Legionella* spp. and other opportunist intracellular pathogens in environmental systems.

Dr Bentham is associate professor in public health microbiology at Flinders University. His research interests include human health risk assessment, *Legionella* spp. ecology, and control and bioremediation of contaminated soils.

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Carriage of *Streptococcus pneumoniae* 3 Years after Start of Vaccination Program, the Netherlands

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To evaluate the effectiveness of the 7-valent pneumococcal conjugate vaccine (PCV7) program, we conducted a cross-sectional observational study on nasopharyngeal carriage of *Streptococcus pneumoniae* 3 years after implementation of the program in the Netherlands. We compared pneumococcal serotypes in 329 prebooster 11-month-old children, 330 fully vaccinated 24-month-old children, and 324 parents with age-matched pre-PCV7 (unvaccinated) controls (ages 12 and 24 months, n = 319 and n = 321, respectively) and 296 of their parents. PCV7 serotype prevalences before and after PCV7 implementation, respectively, were 38% and 8% among 11-month-old children, 36% and 4% among 24-month-old children, and 8% and 1% among parents. Non-PCV7 serotype prevalences were 29% and 39% among 11-month-old children, 30% and 45% among 24-month-old children, and 8% and 15% among parents, respectively; serotypes 11A and 19A were most frequently isolated. PCV7 serotypes were largely replaced by non-PCV7 serotypes. Disappearance of PCV7 serotypes in parents suggests strong transmission reduction through vaccination.

Streptococcus pneumoniae (pneumococcus) is a major cause of respiratory and invasive disease worldwide, particularly in children <5 years of age and elderly persons (*1*). All pneumococcal disease is preceded by nasopharyngeal colonization (*2*). To date, slightly >90 serotypes have been identified. Young children, among whom nasopharyngeal carriage rates are highest, are the main reservoir for pneumococcal spread in families and the community (*3*).

In the United States and other industrialized countries, widespread use of the 7-valent pneumococcal conjugate vaccine (PCV7) (Prevenar; Pfizer, New York, NY, USA) for children has led to a dramatic decline in PCV7-serotype invasive pneumococcal disease (IPD), not only in vaccinated children (*4*) but also in unvaccinated persons of all ages (*5,6*). This indirect effect has substantially contributed to favorable cost-effectiveness estimates of the vaccination program (*7,8*). However, shifts toward nasopharyngeal carriage of non-PCV7 serotypes may eventually counterbalance the direct and indirect benefits of the vaccine, assuming that non-PCV7 serotypes will display similar disease potential (*9–12*). Early evaluation of the effect of pneumococcal vaccination on serotype distribution in disease is hampered by the relative infrequency of IPD and the difficulty of identifying causative agents in respiratory diseases such as pneumonia or in otitis media. Therefore, surveillance of nasopharyngeal carriage of pneumococci in vaccinated and in unvaccinated persons provides another useful tool for monitoring how vaccination affects circulating pneumococcal serotypes.

In the Netherlands, as part of the national immunization program (NIP), vaccination with PCV7 was introduced for all infants born after March 31, 2006, in a 3+1 schedule of vaccinations at 2, 3, 4, and 11 months with no catch-up campaign. To evaluate how this PCV7 vaccination program affected prevalence of pneumococcal serotypes after 3 years, we conducted a cross-sectional observational

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study of nasopharyngeal carriage of pneumococci, and we compared data from vaccinated children and their parents with data from age-matched pre-PCV7 (unvaccinated) controls.

Methods

Study Design

In 2009, after PCV7 vaccination had been conducted for 3 years, we examined nasopharyngeal swabs for pneumococcal carriage from 2 age cohorts: 1) healthy 11-month-old children who had received 3 primary vaccinations according to the Dutch NIP but had not yet received the booster dose at 11 months of age or had had the booster dose within the week before sampling, 2) healthy 24-month-old children vaccinated according to the Dutch NIP. We also examined swabs from 1 parent each for the 24-month-old children. Exclusion criteria for children were known or suspected immunodeficiency, craniofacial or chromosomal abnormalities, coagulation disorders, use of anticoagulant medication, and having older siblings in the household who had received a pneumococcal conjugate vaccine. Parents were excluded if they had a bleeding disorder or used anticoagulant medication. The study (NL24116.000.08) was approved by an acknowledged national ethics committee in the Netherlands. The study was conducted in accordance with the European Statements for Good Clinical Practice.

Data from the vaccinated children cohorts were compared with data from pre-PCV7 control children and their parents derived from a longitudinal, randomized, controlled trial (NCT00189020) that had started in the Netherlands well before national PCV7 implementation for infants. In that trial, children had been included at the age of 6 weeks from July 2005 through February 2006 and were followed-up until 24 months of age. Nasopharyngeal swabs were obtained from children at 12 and 24 months of age and from 1 parent of each of the 24-month-old children; results have been described (*13*).

Nasopharyngeal Swabs

Trained study personnel collected the nasopharyngeal swabs from children and parents by using a flexible, sterile, dry cotton–wool swab transnasally, according to World Health Organization standard procedures (*14*). Also, a transoral nasopharyngeal swab was collected from parents because the pneumococcal yield is known to be higher for adults when both areas are swabbed (*15*). Transoral swabs of the directly observed posterior pharynx were collected on a rigid cotton–wool swab. All swabs in both studies were processed according to the same study procedures by the same laboratory for microbiology as described (*13*). Briefly, swabs were cultured for *S. pneumoniae*, then 1 pneumococcal colony per plate was subcultured and serotyped by the capsular swelling method (Quellung reaction). All serotype 6A isolates were submitted to the National Reference Laboratory of Bacterial Meningitis (Academic Medical Center, Amsterdam) for further discrimination between 6A and the newly discovered serotype 6C by PCR with primers 5106 and 1301 and primers 6C-fwd and 6C-rev (*16*). Results were confirmed by the Quellung reaction with newly available antiserum to identify 6C serotype (Statens Serum Institute, Copenhagen, Denmark). In a post hoc analysis, all serotype 19A isolates from children were examined by the disk-diffusion method for susceptibility to azithromycin, erythromycin, and penicillin and were further tested by Etest (PDM Epsilometer; AB Biodisk, Solna, Sweden) and classified according to Clinical and Laboratory Standards Institute (M100-S20). Because oral antimicrobial drugs are the driving force for resistance in the community, susceptibility to penicillin was further classified according to breakpoints defined by the Clinical and Laboratory Standards Institute; isolates were considered penicillin susceptible (MIC $\leq$0.06 μg/mL), penicillin intermediately resistant (MIC 0.12–1.0 μg/mL), or penicillin resistant (MIC $\geq$2.0 μg/mL) (*17*).

Covariates

A questionnaire, completed by each participant at the time of nasopharyngeal sampling, was used to determine risk factors for nasopharyngeal carriage of pneumococci. The questionnaire asked about the following: age, sex, month of sampling, presence of siblings in the household, daycare attendance, passive smoke exposure indoors, clinical signs of a respiratory tract infection at the time of sampling, antimicrobial drug use within 1 month before the sample was taken, and active smoking of the participating parent.

Statistical Analyses

Data on *S. pneumoniae* carriage were compared with data from the pre-PCV7 control cohort at age 12 months (n = 319) and 24 months (n = 321) and the parents of the 24-month-old children (n = 296) in which children were enrolled as described previously (*13*). According to protocol, the primary study outcome was defined as the prevalence of any of the PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) and any of the non-PCV7 serotypes (all other pneumococcus serotypes including nontypeable isolates) in children. On the basis of previous trial results (*13*), we expected that the smallest difference we would detect would be prevalence of non-PCV7 serotypes at age 11 and 24 months with a 29% carriage rate in the pre-PCV7 control cohort and an estimated 40% in vaccinated children. Therefore, before conducting the study, we estimated that a minimum sample size of ≈330 children in

each group was needed to detect this difference with 80% power with a 2-sided α of 0.05. Differences in prevalence rates were statistically tested by using a 2-sided χ^2 or Fisher exact test, where appropriate. Multivariate analysis with binary logistic regression modeling was used to obtain adjusted estimates of the association between the outcomes and intervention as given by adjusted odds ratios (ORs) and their corresponding 95% confidence intervals (CIs).

Results

Three years after PCV7 implementation in the NIP for all newborns, parents of 11,005 children were invited to participate. A total of 1,045 (9.5%) parents were interested in participating, among which 892 families were assessed for eligibility; 153 families were not assessed because the enrollment target had already been achieved. Of the 892 families, 233 were excluded. The most frequent reason for exclusion was household presence of older siblings who had received a pneumococcal conjugate vaccine (78%). In total, 329 children 11 months of age and 330 children 24 months of age and 1 parent for each of the 24-month-old children (n = 324) were enrolled from February 9 through July 9, 2009.

The mean age of vaccinees was slightly lower (4 weeks at 11 months, and 1 week at 24 months of age; both p<0.001) than that of the pre-PCV7 controls. At 11 months, the proportion of siblings <5 years of age and the exposure to smoking indoors was lower for vaccinees (both p<0.001). At 24 months, the proportion of male participants and the proportion of children who had received antimicrobial drugs within 1 month before the swab was taken was higher among vaccinees (both p = 0.03). Also, the months of sampling differed between both studies (p<0.001; Table 1).

Carriage of *S. pneumoniae* in Children

Prevalence of any of the PCV7 serotypes was 38% among the pre-PCV7 controls compared with 8% among prebooster children at 11 months (OR 0.13, 95% CI 0.08–0.21) and 36% among the pre-PCV7 controls compared with 4% among children 24 months of age (OR 0.08, 95% CI 0.05–0.14) (Table 2). Prevalence rates for individual PCV7 serotypes were significantly lower among vaccinees except for the infrequently carried serotypes 18C (p = 0.45) and 4 (p = 0.49), which were almost absent before and after introduction of PCV7. Among children 24 months of age, serotype 6B had remained in only 2% of all vaccinated children, serotypes 19F and 18C in 1%, and all other PCV7 serotypes had almost disappeared (Table 3).

In contrast, corresponding prevalences of non-PCV7 serotypes were 29% and 39% among children 11 months of age (OR 1.59, 95% CI 1.15–2.21) and 30% and 45% among children 24 months of age (OR 1.88, 95% CI 1.36–2.59) in the pre-PCV7 and post-PCV7 cohorts, respectively (Table 2). In prebooster children, serotype 19A had become the most prevalent (10%), serotype 11A had remained stable at ≈4%, followed by 6A and 15B (each 3%). At 24 months, serotype 11A had become the most prevalent serotype (7%), followed closely by 19A (6%); proportions of each of these serotypes had doubled among the 24-month-old

Table 1. Characteristics of children and their parents before and 3 years after implementation of PCV7 vaccination program, the Netherlands*

	11-mo-old children			24-mo-old children			Parents		
Characteristic	Pre-PCV7, n = 319	Post-PCV7, n = 329†	p value‡	Pre-PCV7, n = 321	Post-PCV7, n = 330	p value‡	Controls, n = 296	Vaccinees, n = 324	p value‡
Male	156 (49)	181 (55)	0.12	155 (48)	187 (57)	0.03	51 (17)	53 (16)	0.76
Mean age (SD)	12.0 mo (0.3)	11.0 mo (0.3)	<0.001§	24.2 mo (0.6)	24.0 mo (0.3)	<0.001§	34.7 y (4.9)	35.1 y (4.4)	0.27§
Presence of siblings <5 y of age	126 (40)	84 (26)	<0.001	127 (40)	135 (41)	0.73	NA	NA	NA
Day care attendance¶	208 (65)	226 (69)	0.35	224 (70)	233 (71)	0.82	NA	NA	NA
Passive smoke exposure#	21 (7)	5 (2)	0.001	26 (8)	16 (5)	0.09	NA	NA	NA
Signs of RTI**	95 (30)	95 (29)	0.80	82 (26)	69 (21)	0.16	NA	NA	NA
Antimicrobial drug use††	20 (6)	24 (7)	0.60	10 (3)	23 (7)	0.03	9 (3)	20 (6)	0.07
Period of sampling									
Oct–Mar	149 (47)	82 (25)	<0.001	156 (48)	86 (26)	<0.001	NA	NA	NA
Apr–Sep	170 (53)	247 (75)	NA	166 (52)	244 (74)	NA	NA	NA	NA
Active smoking	NA	NA	NA	NA	NA	NA	40 (14)	34 (10)	0.25

*Values are no. (%) except as indicated. PCV7, 7-valent pneumococcal conjugate vaccine; RTI, respiratory tract infection; NA, not applicable.
†Swabs taken just before booster vaccination at 11 mo of age or within 1 week after booster vaccination.
‡By χ^2 test or Fisher exact test (2-sided) where appropriate.
§By independent-samples *t* test.
¶Defined as >4 h/wk with ≥1 child from a different household.
#Defined as passive tobacco smoke exposure indoors to ≥1 cigar or cigarette during ≥5 d/wk.
**Defined by evaluation of parents.
††Defined as use of oral or intravenous antibiotics within 1 mo before sample was taken.

Table 2. Frequencies of nasopharyngeal carriage of *Streptococcus pneumoniae* in children and their parents before and 3 years after implementation of PCV7 vaccination program, the Netherlands*

Participant s	Pre-PCV7, no. (%)	Post-PCV7, no. (%)†	OR‡ (95% CI)	aOR§ (95% CI)
11-mo-old children	n = 319	n = 329		
PCV7	122 (38)	25 (8)	0.13 (0.08–0.21)	0.14 (0.09–0.23)
Non-PCV7	92 (29)	129 (39)	1.59 (1.15–2.21)	1.64 (1.15–2.32)
All	214 (67)	154 (47)	0.43 (0.31–0.59)	0.44 (0.31–0.63)
24-mo-old children	n = 321	n = 330		
PCV7	114 (36)	14 (4)	0.08 (0.05–0.14)	0.08 (0.05–0.15)
Non-PCV7	97 (30)	148 (45)	1.88 (1.36–2.59)	2.01 (1.43–2.84)
All	211 (66)	162 (49)	0.50 (0.37–0.69)	0.51 (0.36–0.72)
Parents	n = 296	n = 324		
PCV7	25 (8)	2 (1)	0.07 (0.02–0.29)	0.06 (0.01–0.26)
Non-PCV7	25 (8)	49 (15)	1.93 (1.16–3.22)	1.98 (1.16–3.37)
All	50 (17)	51 (16)	0.92 (0.60–1.41)	0.90 (0.57–1.40)

*PCV7, all serotypes included in 7-valent pneumococcal conjugate vaccine; OR, odds ratio; CI, confidence interval; aOR; adjusted odds ratio; non-PCV7, all other serotypes not included in 7-valent pneumococcal conjugate vaccine; all; all pneumococcal serotypes.
†325/329 (99%) swabs are taken just before the booster vaccination at 11 mo of age and 4/329 (1%) children had received a booster vaccination within 1 week before the sample was obtained.
‡All ORs are based on comparison with pre-PCV7 control cohort.
§For children, ORs were adjusted by multivariate analysis for sex, month of sampling, presence of siblings in the household, day care attendance, passive smoke exposure indoors, symptoms of a respiratory tract infection during sampling, and antimicrobial drug use within 1 mo before the sample was taken. For parents, ORs were adjusted for sex, months of sampling, antimicrobial drug use within 1 month before the sample was taken, and active smoking.

cohort compared with the pre-PCV7 control cohort. Among children 11 and 24 months of age, serotype 6A had declined while serotype 6C had increased, but the numbers were too small for statistical significance (Table 3). The overall prevalence rate for carriage of *S. pneumoniae* among the pre-PCV7 controls was 67% compared with 47% among prebooster children at 11 months of age (OR 0.43, 95% CI 0.31–0.59) and 66% compared with 49% at 24 months of age (OR 0.50, 95% CI 0.37–0.69), respectively (Table 2).

Susceptibility testing of all serotype 19A isolates showed that no isolates were penicillin resistant. Among the pre-PCV7 controls and among vaccinated children, respectively, 0 and 3 (6%) isolates were intermediately resistant to penicillin, and 0 and 2 isolates (4%) were nonsusceptible to azythromycin and to erythromycin.

Carriage of *S. pneumoniae* in Parents

Prevalence of any of the PCV7 serotypes was 8% among parents of 24-month-old children in the pre-PCV7 controls and 1% among parents of vaccinated children 3 years after PCV7 implementation (OR 0.07, 95% CI 0.02–0.29). Corresponding figures for non-PCV7 serotypes were 8% and 15% (OR 1.93, 95% CI 1.16–3.22), respectively. A stable pneumococcal prevalence rate of 17% and 16% was observed among parents (OR 0.92, 95% CI 0.60–1.41), respectively (Table 2). Before PCV7 implementation, the most frequently found pneumococcal serotypes among parents had been 19F (3%), 14, and 6B (each 2%). After PCV7 implementation, serotypes 11A (2%) and 19A (2%) were the most frequently isolated, and serotype19F was the only PCV7-included serotype recovered, albeit in only 2 of 324 parents (Table 3).

Multivariate Analysis of Covariates

Unadjusted associations are shown as ORs in children and parents (Table 2). Multivariate analysis showed that associations after adjustments for some potential confounders did not differ from the unadjusted associations.

Discussion

Three years after NIP implementation of PCV7 for all newborns in the Netherlands, PCV7-serotype carriage of *S. pneumoniae* was reduced 80%–90% among vaccinated children at 11 and 24 months of age. Among parents of vaccinated children, carriage of PCV7 serotypes had almost disappeared. This impressive reduction of PCV7-serotype carriage in infants is larger than that observed in clinical trials, which showed 50%–60% reduction in PCV7 rates after conjugate vaccination, and should be attributed to herd effects (*13*,*18,19*). Herd effects would also account for the disappearance of PCV7 serotypes in parents. This large effect might in part be a result of a high pneumococcal vaccine uptake because 94.4% of all 2-year-old children in the Netherlands have been fully vaccinated (*20*). Our data confirm the major role of young infants in the transmission of pneumococci in the community.

Herd effects may also have contributed to the reported unexpectedly high reductions of otitis media (by 43%) (*21*) and all-cause pneumonia (by 33%) (*22*) in young children in the United States since PCV7 introduction. These reductions exceed overall vaccine efficacy found in randomized controlled trials: 6%–9% reduction of otitis media (*9,23*), and 4% reduction of all-cause pneumonia (*24*). However, a recent US study on community-acquired pneumonia (with radiographic confirmation) found no

consistent reductions in pneumonia rates among children and adults, except for children <1 year of age (*25*). Whether this finding is the result of replacement disease by other nonvaccine pneumococcal serotypes, other pathogens, or other causes remains to be evaluated. Nasopharyngeal serotype replacement remains a potential drawback of vaccination with pneumococcal conjugate vaccines.

Increased rates of carriage of nonvaccine serotypes were also observed in this study. In vaccinated infants and their parents; serotypes 19A and 11A were the most frequently carried serotypes in the Netherlands. In the United States, multidrug resistant serotype 19A has become a frequent cause of IPD as well as of otitis media in children (*26,27*). There is ongoing debate about the actual role of PCV7 introduction and the increase in serotype 19A; antimicrobial drug pressure and secular trends have been emphasized (*28*). In our study, however, post hoc susceptibility testing of all 19A isolates showed a low prevalence of nonsusceptible strains among controls and vaccinees. In addition, our group previously reported a significant increase in serotype 19A carriage after PCV7 vaccinations in a study conducted in a randomized controlled setting (*29*), excluding secular trends and indicating a direct role of PCV7. A trend toward lower carriage rates of serotype 6A and higher carriage rates of serotype 6C was observed in both age groups, suggesting PCV7 cross-protection for serotype 6A but not for serotype 6C, in line with other carriage studies (*30*). However, serotypes 11A and 6C have not yet been reported as a frequent cause of IPD in the Netherlands (*12*).

Observed changes in prevalence of serotype carriage may not be entirely random but may be directly related to the serotype capsule size, which in turn is related to the polysaccharide composition and metabolic costs of the capsule for the bacterium (*31*). Pneumococci with larger capsules are more resistant against nonopsonic phagocytosis and more commonly colonize young children. Our results agree with results of Weinberger et al., which show a significant increase in carriage of highly encapsulated serotypes such as 19A, 11A, 10A, and 35F (*31*). Furthermore, the serotype-specific capsule has been shown to be a major factor in the potential to cause IPD, independent of genetic background and temporal or

Table 3. Frequencies of nasopharyngeal carriage of individual *Streptococcus pneumoniae* serotypes in children and their parents before and 3 years after implementation of PCV7 vaccination program, the Netherlands*

	11-mo-old children			24-mo-old children			Parents		
Serotype	Pre-PCV7, n = 319	Post-PCV7, n = 329†	p value‡	Pre-PCV7, n = 321	Post-PCV7, n = 330	p value‡	Controls, n = 296	Vaccinees, n = 324	p value‡
PCV7									
19F	36 (11)	5 (2)	<0.001	24 (8)	4 (1)	<0.001	9 (3)	2 (1)	0.02
23F	34 (11)	6 (2)	<0.001	28 (9)	0 (0)	<0.001	2 (1)	0 (0)	0.14
6B	26 (8)	12 (4)	0.02	43 (13)	7 (2)	<0.001	5 (2)	0 (0)	0.02
14	10 (3)	0 (0)	0.001	8 (3)	0 (0)	0.003	6 (2)	0 (0)	0.01
9V	9 (3)	1 (0)	0.01	6 (2)	1 (0)	0.07	1 (0)	0 (0)	0.30
18C	6 (2)	1 (0)	0.07	4 (1)	2 (1)	0.45	0 (0)	0 (0)	NA
4	1(0)	0 (0)	0.49	1 (0)	0 (0)	0.49	2 (1)	0 (0)	0.14
Non-PCV7§									
19A	5 (2)	32 (10)	<0.001	9 (3)	21 (6)	0.03	4 (1)¶	6 (2)	0.62
11A	11 (3)	12 (4)	0.89	10 (3)	22 (7)	0.04	3 (1)	8 (2)	0.17
6A#	19 (6)	11 (3)	0.11	17 (5)	9 (3)	0.09	2 (1)	2 (1)	0.93
15B	3 (1)	10 (3)	0.06	8 (3)	6 (2)	0.55	1 (0)	2 (1)	0.62
15C	4 (1)	4 (1)	0.97	2 (1)	8 (2)	0.06	1 (0)	2 (1)	0.62
6C	5 (2)	8 (2)	0.43	5 (2)	10 (3)	0.21	1 (0)	1 (0)?	0.95
22F	4 (1)	7 (2)	0.39	2 (1)	5 (2)	0.45	0 (0)	2 (1)	0.18
10A	1 (0)	6 (2)	0.12	1 (0)	8 (2)	0.04	1 (0)	2 (1)	0.62
16F	1 (0)	6 (2)	0.12	4 (1)	6 (2)	0.75	3 (1)	1 (0)	0.27
23B	5 (2)	5 (2)	1,00	12 (4)	11 (3)	0.78	0 (0)	4 (1)	0.06
35F	2 (1)	5 (2)	0.45	0 (0)	9 (3)	0.004	0 (0)	3 (1)	0.10
NT	1 (0)	6 (2)	0.12	3 (1)	5 (2)	0.73	1 (0)	4 (1)	0.21
Other	31 (10)	17 (6)	NA	21 (7)	26 (8)	NA	8 (3)	12 (4)	NA

*Values are no. (%) except as indicated. PCV7, all serotypes included in the 7-valent conjugate vaccine; NA, not applicable; non-PCV7, all other serotypes not included in the 7-valent conjugate vaccine; NT, nontypeable.
†325/329 (99%) swabs were taken just before the booster vaccination at 11 mo of age, and 4/329 (1%) children had received a booster vaccination within 1 wk before the sample was obtained.
‡All p values are for comparison with control group and calculated with χ^2 or 2-tailed Fisher exact test where appropriate.
§Only non-PCV7 serotypes with >5 isolates in 11- or 24-mo-old children or in parents are included in this table.
¶In only 1 parent, pneumococci were present in both samples but with detection of a different serotype; serotype 19A was found in the transnasal swab and serotype 3 was found in the transoral swab. Serotype 19A is included in this table.
#After discrimination between 6A and 6C by PCR, different serotypes were found by PCR compared with Quellung: 3 isolates (serotypes 6B [n = 1] and 14 [n = 2]) in 24-mo-old controls and 2 isolates (serotypes 11 and 15) in 24-mo-old vaccinees. These serotypes were not included in this table.

geographic settings (*32*). This serotype-specific difference in disease potential has also been shown for mucosal infections, although the differences between serotypes were less apparent compared with differences in invasive potential (*33*). In addition, serotypes are independently associated with IPD severity (*34,35*). Harboe et al. showed that highly encapsulated and frequently carried serotypes such as 11A, 10A, and 19A have high mortality rates among healthy persons >5 years of age that are comparable to PCV7 serotypes such as 19F or 6B. Therefore, replacing serotypes in carriage may potentially cause equally severe disease. Furthermore, frequently carried serotypes are more likely to affect patients with concurrent illnesses than are infrequently carried serotypes with high invasive disease potential such as serotypes 1 and 7F (*36*). Replacement with highly encapsulated pneumococci may therefore substantially reduce vaccine benefits, especially for those who are older or have chronic disease (*37*). However, in addition to the prevalence in carriage and the disease potential of the serotype, the prevalence and severity of pneumococcal disease are also associated with genetic background and presence of drug-resistant clones (*38*) and depends on population or patient characteristics (*36*). As a consequence, it is crucial to monitor and critically evaluate all of these aforementioned aspects.

In contrast to several other carriage studies (*11*), our study found a significant reduction (20% at 11 months and 17% at 24 months of age) in overall pneumococcal carriage 3 years after the PC7 vaccination program began. Previously, we reported a 10% decline in overall pneumococcal carriage after PCV7 vaccination in a randomized controlled trial setting in the Netherlands after reduced-dose schedules and before implementation of PCV7 in the national immunization program for children (*13*). Herd effects may have contributed to the larger reduction in PCV7-serotype and overall pneumococcal carriage in the present surveillance study. We must, however, be cautious about ascribing the reported reduction in overall pneumococcal carriage in children to the introduction of PCV7 because this was an observational study and other unmeasured factors such as viral infections, seasonal variations, and temporal trends could not be taken into account (*39*). Among parents, overall pneumococcal carriage did not change, but low carriage rates in adults make it harder to detect significant changes. Because the same study procedures were followed by the same well-trained research nurse team and laboratory personnel in both studies, we do not think that the observed carriage reduction is an artifact.

For evaluation of our study results, some potential limitations should be taken into account. First, because our data are observational, we showed associations and no causalities between the introduction of PCV7 and changes in pneumococcal colonization. Although several potential confounders were measured and appeared to differ between the comparison groups, multivariate analysis showed that our results were quite robust. Second, the postvaccination data came from a cross-sectional cohort study including 2 separate age groups, whereas the pre-PCV7 control data were derived from a longitudinal study in which data were collected from the same children at age 12 and 24 months. However, we previously found that potential within-person dependency was not substantially affecting these carriage data, probably because of the large interval between carriage sample collections (*13*). Third, the study was not adequately powered to evaluate serotype-specific differences. Lastly, we used a single-colony method for serotyping in both studies. Currently, improved techniques for detection of multiserotype carriage, e.g., the newer, more sensitive PCRs, are available. Multiple serotype carriage methods might have revealed more nonvaccine strains in both studies, pointing to unmasking instead of true replacement after eradication of vaccine strains. The strengths of our study are the relatively high carriage rates found in both studies in the Netherlands compared with other Western countries, a high PCV7 uptake in the NIP, and the possibility to evaluate the effect of vaccination with PCV7 on pneumococcal carriage in adult contacts. Also, drug-resistant clones do not confound the results because antimicrobial drug use and consequent resistance are low in the Netherlands compared with other European countries (*40*).

Since 2009, pneumococcal vaccines with broader coverage have been licensed and will be introduced into vaccination programs worldwide. The effects of these broader coverage vaccines on potential shifts in pneumococcal serotypes in the nasopharynx are still largely unknown. To predict the long-term health and economic effects, close monitoring is warranted.

In conclusion, 3 years of vaccination with PCV7 has led to impressive shifts in serotype-specific carriage of *S. pneumoniae* in children and their parents. This finding indicates a major role of infants in transmission of pneumococci in the population.

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Nosocomial Pandemic (H1N1) 2009, United Kingdom, 2009–2010

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To determine clinical characteristics of patients hospitalized in the United Kingdom with pandemic (H1N1) 2009, we studied 1,520 patients in 75 National Health Service hospitals. We characterized patients who acquired influenza nosocomially during the pandemic (H1N1) 2009 outbreak. Of 30 patients, 12 (80%) of 15 adults and 14 (93%) of 15 children had serious underlying illnesses. Only 12 (57%) of 21 patients who received antiviral therapy did so within 48 hours after symptom onset, but 53% needed escalated care or mechanical ventilation; 8 (27%) of 30 died. Despite national guidelines and standardized infection control procedures, nosocomial transmission remains a problem when influenza is prevalent. Health care workers should be routinely offered influenza vaccine, and vaccination should be prioritized for all patients at high risk. Staff should remain alert to the possibility of influenza in patients with complex clinical problems and be ready to institute antiviral therapy while awaiting diagnosis during influenza outbreaks.

Nosocomial influenza is a well-recognized problem in acute-care hospital settings (*1,2*). Outbreaks of influenza A have been reported in general wards (*3,4*), pediatric units (*5*), neonatal intensive care units (ICUs) (*6–8*), hemopoietic and solid organ transplantation units (*9–11*), oncology and neurology units (*12,13*), and facilities for the elderly and for long-term care (*14–17*). Associated illness and death rates are particularly high in immunocompromised patients (*18–20*).

On June 11, 2009, the World Health Organization reported the first influenza pandemic of the 21st century (*21,22*). Although most cases of pandemic (H1N1) 2009 have been mild or subclinical, patients with severe disease have considerably affected hospital systems (*23*). Three nosocomial outbreaks of pandemic (H1N1) 2009 were reported in hemopoietic transplantation units and oncology wards. One outbreak was reportedly mild (*24*), and the other 2 involved aggressive illness, severe complications, and deaths (*25,26*).

In addition to outbreaks of nosocomial influenza, sporadic nosocomial influenza infections also occur but generally are not reported in the literature. We describe the clinical and epidemiologic characteristics of nosocomial pandemic (H1N1) 2009 infections during 2009–2010 in the United Kingdom that were identified during surveillance rather than through outbreak control activity.

Methods

During the pandemic (H1N1) 2009 outbreak in the United Kingdom, the Influenza Clinical Information Network (FLU-CIN) collected clinical and epidemiologic data for patients with virologically confirmed pandemic (H1N1) 2009 virus infection admitted to hospitals (*27*). Data included demography, symptoms, medical history, influenza vaccination history, relevant timelines, investigations and results, treatment (e.g., antiviral and antibacterial drugs), outcome, and cause of death when available. Trained health care workers abstracted data from

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case notes. During May 11, 2009–January 31, 2010, data were accrued from 75 National Health Service hospitals in 31 cities or towns in England, Scotland, Wales, and Northern Ireland.

From this source cohort, we defined patients with nosocomial pandemic (H1N1) 2009 as those admitted to a hospital for a reason other than acute respiratory infection in whom respiratory symptoms developed ≥72 hours (3 days) after admission. In addition, we included infants who had not left the hospital since birth in whom pandemic (H1N1) 2009 had developed. We included transfers from other hospitals when a transfer was for a reason other than influenza and when the history of influenza clearly indicated that it had been acquired at another hospital. FLU-CIN procedures were reviewed and approved by the Ethics and Confidentiality Committee of the National Information Governance Board for Health and Social Care in England for collection, storage, and use of personal data for surveillance purposes.

Results

Of 1,520 patients in the FLU-CIN cohort, illnesses in 30 (2.0%) (15 children) met the criteria for nosocomial influenza (Tables 1, 2 [adults] and Tables 3, 4 [children]). Patient ages ranged from 41 days to 76 years at onset of influenza symptoms (median age 44 years for adults and 1 year for children).

Concurrent Conditions and Reasons for Admission

Twelve (80%) adults and 14 (93%) children had serious underlying illnesses. The most common illnesses were hematologic malignancy for adults (5), and congenital abnormality or prematurity (7) or malignancy (4) for children.

Of the 15 adults, 2 had been admitted for elective surgical procedures; 1 for emergency surgery; and 8 for deterioration of chronic conditions, including complications caused by chemotherapy, malignancy, or transplantation. Two patients were admitted for pancreatitis (1 of whom had underlying myeloma); 1 patient was admitted for obstetric complications, and another patient was admitted for psoriasis. Of 15 children, 3 were admitted for elective procedures, 6 had been in the hospital since birth (because of prematurity or congenital abnormality), 1 was transferred from another hospital, and 5 had acute conditions (Table 2).

Pandemic Vaccination Status and Use of Antiviral and Antibacterial Drugs

None of the patients had received pandemic influenza vaccine. Although 14 adults were eligible because of concurrent conditions, influenza symptoms developed in 11 either before vaccine became available or before they would have seroconverted if vaccinated at the earliest opportunity (vaccine became available in the United Kingdom at the end of October 2009). Four children were eligible because of age and concurrent conditions, and symptoms developed in 3 before vaccine became available or before they would have seroconverted. Only 2 patients (both adults) had received seasonal influenza vaccine.

Twenty-one (72%) of 29 patients (10 children) received antiviral medication as inpatients (data were unknown for 1 patient); all initially received oseltamivir as monotherapy. Therapy for 2 patients (nos. 13 and 18) was switched from oseltamivir to zanamivir after 4 days and 10 days, respectively, because drug-resistant virus carrying the H275Y mutation was identified. Administration of

Table 1. Characteristics of 15 hospitalized adults with nosocomial pandemic (H1N1) 2009, United Kingdom, 2009–2010

Patient no.	Age, y/sex	Reason for admission	Main underlying illnesses	Signs and symptoms
1	51/F	Pancreatitis	None recorded	Fever, unknown data in other fields
2	44/M	Transplant complications	Lymphoma	Productive cough, headache, coryza, myalgia
3	34/M	Emergency surgery	Diabetes	Unknown
4	18/F	Elective surgery	Neurodegenerative disease	Dyspnea, malaise
5	48/M	Chemotherapy	Hematologic malignancy	Fever, sore throat
6	43/M	Pancreatitis	Chronic liver disease	Fever, malaise, myalgia
7	51/M	Not recorded	Lymphoma	Fever, dry cough, diarrhea, myalgia, arthralgia
8	39/F	Metastatic soft tissue	Malignancy	Dry cough, dyspnea
9	76/M	Elective surgery	Diabetes, heart disease	Productive cough, diarrhea, dyspnea
10	45/F	Not stated	Myeloma	Fever, productive cough, nausea, anorexia, malaise
11	44/F	Psoriasis	Psoriasis	Fever, unknown data in other fields
12	22/M	Posttransplant complications	Renal transplant, congenital abnormalities	Fever, cough, anorexia, malaise
13	52/M	Elective procedure	Lymphocytic leukemia	Dyspnea, altered consciousness
14	33/F	Obstetric complications	None recorded	Fever, productive cough
15	60/M	Cerebrovascular disease	Diabetes, obesity	Unknown

Table 2. Timelines and outcomes for 15 hospitalized adults with nosocomial pandemic (H1N1) 2009, United Kingdom, 2009–2010

Patient no.	Age, y/sex	Duration, d		Maximum level of care*	Outcome†
		Hospital admission to symptom onset	Symptom onset to receipt of antiviral therapy		
1	51/F	26	0	0/1	Unknown data
2	44/M	14	0	0/1	Recovered
3	34/M	8	0	3	Died
4	18/F	4	Not given	2	Transferred to other hospital
5	48/M	9	4	0/1	Recovered
6	43/M	5	0	0/1	Recovered
7	51/M	29	0	3	Died
8	39/F	5	3	0/1	Died
9	76/M	11	Not given	3	Died
10	45/F	24	2	0/1	Recovered
11	44/F	14	Not given	3	Transferred, improved
12	22/M	5	0	3	Died
13	52/M	78	1‡	3	Recovered
14	33/F	7	3	0/1	Recovered
15	60/M	13	Not given	3	Recovered

*Level 0 care is given to patients whose care needs can be met through normal ward care. Level 1 care is given to patients at risk for a deteriorating condition or recently relocated from higher levels of care whose needs can be met in an acute-care ward with additional advice and support from the critical-care team. Level 3 care is given to patients requiring advanced respiratory support alone or basic respiratory support and support for ≥2 organ systems; this level includes all patients with complex conditions that required support for multiorgan failure (intensive care unit). Level 2 care is given to patients requiring more detailed observation or intervention, including support for a single failing organ system and those changing from higher levels of care (high dependency unit).
†Deaths were attributed to pandemic (H1N1) 2009.
‡Oseltamivir was replaced with zanamivir on day 5 because of identification of the H275Y drug-resistance mutation.

antiviral drugs ranged from 0 to 8 days after symptom onset; 12 (57%) of 21 patients who received therapy did so within 48 hours. Sixteen patients were already receiving antibacterial drugs when influenza symptoms began. Two of these patients had a bacterial co-infection: coagulase-negative staphylococci in a blood culture for 1 patient and *Pseudomonas aeruginosa* in an unspecified intravenous line in 1 patient. Twelve patients received antibacterial drugs during their respiratory illness, 2 of whom had *Haemophilus influenzae* in sputum samples and 1 (co-infected with rhinovirus) who had had a blood culture positive for *Klebsiella* sp.

Signs and Symptoms

The most common signs were fever (8 [53%] adults and 12 [80%] children), cough (10, mostly adults), coryza (8, mostly children), and dyspnea (7). Fewer patients had malaise (4); myalgia (3); anorexia, nausea,

Table 3. Characteristics of 15 hospitalized children with nosocomial pandemic (H1N1) 2009, United Kingdom, 2009–2010

Patient no.	Age/sex	Reason for admission	Main underlying illnesses	Signs and symptoms
16	12 y/F	Elective surgery	Heart disease	Fever, unknown data in other fields
17	2 y/M	Malignancy	Malignancy	Dry cough, coryza
18	4 y/F	Bone marrow aspirate	Acute myeloid leukemia	Fever, productive cough
19	15 y/M	Ulcerative colitis	Ulcerative colitis	Fever, unknown data in other fields
20	123 d/F	Inpatient care from birth	Prematurity	Fever, dyspnea
21	1 y/F	Laryngomalacia, transfer from tertiary care center	Genetic disorder	Fever, dyspnea
22	1 y/M	Investigation	Acute lymphoblastic leukemia	Fever, dry cough, coryza
23	9 y/M	Sepsis	Cerebral palsy, septic pressure sore	Fever, patient sedated and ventilated
24	12 y/M	Anorexia	Anorexia	Fever, coryza, nausea, sneezing
25	82 d/M	Inpatient care from birth	Congenital abnormalities	Coryza, dyspnoea
26	64 d/M	Inpatient care from birth	Congenital abnormalities	Fever, coryza
27	151 d/M	Prematurity	Prematurity	Fever, coryza
28	101 d/F	Inpatient care from birth	Congenital abnormalities	Fever, coryza
29	41 d/F	Inpatient care from birth	Cystic fibrosis	Fever, rash
30	9 y/F	Elective surgery	Hematologic malignancy	Fever

Table 4. Timelines and outcome for 15 hospitalized children with nosocomial pandemic (H1N1) 2009, United Kingdom, 2009–2010

Patient no.	Age/sex	Duration, d		Maximum level of care*	Outcome
		Hospital admission to symptom onset	Symptom onset to receipt of antiviral therapy		
16	12 y/F	10	Not given	3	Recovered
17	2 y/M	24	Not given	0/1	Died at home
18	4 y/F	54	2†	0/1	Recovered
19	15 y/M	11	8	0/1	Recovered
20	123 d/F	123‡	Unknown data	3	Died after transfer to another hospital
21	1 y/F	14	1	0/1	Recovered
22	1 y/M	6	5	3	Recovered
23	9 y/M	Unknown (transferred)	3	3	Recovered
24	12 y/M	14	Not given	0/1	Recovered
25	82 d/M	82‡	1	3	Died§
26	64 d/M	64‡	Not given	1	Recovered
27	151 d/M	151‡	3	3	Recovered
28	101 d/F	101‡	1	3	Recovered
29	41 d/F	41‡	1	3	Recovered
30	9 y/F	12	1	0/1	Recovered

*Level 3 care is given to patients requiring advanced respiratory support alone or basic respiratory support and support for ≥2 organ systems; this level includes all patients with complex conditions that required support for multiorgan failure (intensive care unit). Level 0 care is given to patients whose care needs can be met through normal ward care. Level 1 care is given to patients at risk for a deteriorating condition or recently relocated from higher levels of care whose needs can be met in an acute-care ward with additional advice and support from the critical-care team.
†Oseltamivir was replaced with zanamivir on day 11 because of identification of the H275Y drug-resistant mutation (patient also received acyclovir throughout hospitalization);
‡Inpatient since birth.
§Attributed to pandemic (H1N1) 2009.

diarrhea (2 each); and arthralgia, sore throat, headache, vomiting, altered consciousness, sneezing, and rash (a child) (1 each).

Course of Illness

Median length of hospitalization before onset of influenza symptoms was 11 days for adults (range 4–78 days) and 13 days (range 6–54 days) for children, excluding infants in a hospital since birth. For infants in a hospital since birth, the interval from birth to onset of influenza signs ranged from 41 to 123 days (median 78 days).

Results of chest radiography ≤3 days after onset of influenza symptoms were documented for 8 adults and 5 children (43%). Of these patients, 4 adults and 1 child (38%) had radiologically confirmed pneumonia.

Level 0 is care given to patients whose care needs can be met through normal ward care. Level 1 care is given to patients at risk for a deteriorating condition or recently relocated from higher levels of care whose needs can be met in an acute-care ward with additional advice and support from the critical-care team. Level 2 care is given to patients requiring more detailed observation or intervention, including support for a single failing organ system and those changing from higher levels of care (high dependency unit). Level 3 care is given to patients requiring advanced respiratory support alone or basic respiratory support and support for ≥2 organ systems. This level includes all patients with complex conditions requiring support for multiorgan failure (ICU).

Seven adults and 8 children (50%) required level 3 care (ICU, pediatric ICU, or neonatal ICU). One (3%) adult required level 2 care. Six adults required mechanical ventilation and 1 required noninvasive ventilation (data for ventilatory support were unknown for 1 adult). Three children required mechanical ventilation and 1 required noninvasive ventilation. The remaining 4 children who received level 3 care were 3 infants and 1 child, each of whom required a period of close monitoring, but did not ultimately require ventilation. The remaining 7 adults and 7 children required level 0 or 1 care.

Outcomes and Mortality Rates

Five (33%) of 15 adults died in the acute-care hospital that provided treatment, 2 within 30 days after symptom onset. Of adults who died, 3 had underlying malignancy (1 noted to be terminal) or were immunocompromised and 2 had diabetes (type I and type II respectively). Pandemic (H1N1) 2009 was included in the recorded causes of death for all 5 adults. Although some patients had a prolonged hospital stay of ≤7 months, all remaining adults recovered from influenza and were discharged from the hospital.

Of 15 children, 3 (20%) were known to have died, although only 1 (a neonate with multiple congenital problems) died at the hospital where surveillance was conducted; acute respiratory distress syndrome/lower respiratory tract infection was stated as a cause of death. Another child, with malignancy, whose death was expected, died at home shortly after discharge. The third

child was transferred to another hospital, and cause of death is unknown. All other children recovered from pandemic (H1N1) 2009. Two children remained in the hospital for treatment of their underlying malignancy, and the other children were discharged.

Discussion

Although pandemic (H1N1) 2009 produced a generally mild illness, in the United Kingdom, as elsewhere, severe illness developed in a small proportion of relatively young patients who required hospitalization (*28*). Although nosocomial outbreaks of pandemic (H1N1) 2009 have been described, (*24–26*) sporadic nosocomial cases of pandemic (H1N1) 2009 identified during surveillance activities have not been described. The present case series has the advantage of being derived from a larger cohort of hospital inpatients in whom confirmation of pandemic (H1N1) 2009 was obtained by using nationally standardized PCR criteria, from settings where clinical management and infection control precautions were driven by national guidelines (*29,30*), and with data abstracted by trained nurses (*27*).

We based our definition of nosocomial influenza on a recent study of health care–associated influenza in children (*31*). A recent systematic review of incubation periods of acute respiratory viral infections found that the median incubation period for influenza was 1.4 days for influenza A, and symptoms developed in 95% of patients in ≤2.8 days (*32*). These findings suggest that our cutoff point, 3 days after admission, make inclusion of community cases unlikely. In addition, in no patients did onset of respiratory illness occur <4 days after hospital admission; median length of hospitalization before symptom onset was 11 days for adults and 13 days for infants. Therefore, inadvertent inclusion of community-acquired cases is highly unlikely.

On the basis of information obtained in the study, we cannot determine where and from whom patients acquired influenza. However, 3 routes are possible. First, infection could have been acquired from other patients; 1 patient shared a bay with a patient who was presymptomatic at the time but for whom influenza was diagnosed 1 day later. Second, transmission from visitors of patients cannot be ruled out. Although national guidelines strongly discourage persons with influenza-like symptoms from visiting patients (*29*), this recommendation may have been difficult to implement, particularly for parents of sick children who often provide most hands-on care in a hospital. Third, transmission may have occurred from an infectious health care worker (because staff continue to work when infected with influenza [*33*]) or from contaminated hands of a health care worker. Transmission from asymptomatic persons might occur in all 3 instances (*34*).

Nosocomial cases in this study occurred equally in adults and children. Consistent with previous findings (*3*), most patients had ≥1 serious underlying illnesses, notably hematologic malignancies, congenital disorders, or prematurity. Staff and caregivers of patients with hematologic malignancy and prematurity are often particularly vigilant for symptoms suggestive of infectious disease. Although we detected nosocomial influenza in patients admitted to nonmedical areas (for emergency or elective surgery), many cases of nosocomial infection in other patient groups probably have been overlooked, particularly because influenza in these groups is likely to have been milder. Additionally, some patients are likely to have been discharged from a hospital during the incubation period of nosocomially acquired pandemic influenza. Thus, in this case series, detecting such patients would not have been possible.

More than half of patients required level 2 or level 3 care, which is higher than that required by the source cohort (12%) (*27*). Approximately one fifth of children and one third of adults died. Although the deaths of 2 patients were expected because of the stage of their underlying malignancy, this case-fatality rate is far higher than that for patients with pandemic (H1N1) 2009 and concurrent conditions in the source cohort (5%) (*27*). The combined factors of increased host susceptibility (*18–20*), prolonged virus shedding in immunocompromised children (*35*), and increased likelihood of development of drug resistance (*36*) raise questions about the need for enhanced infection control procedures in special-care–infant units, pediatric wards, and hemopoietic transplant units and a requirement that staff working in these areas be vaccinated (*37–39*). Precautions should include restricting unnecessary movement of patients to units with particularly vulnerable patients and postponement of semi-elective (nonurgent) procedures for hematology patients during peak pandemic activity.

Vaccine against pandemic (H1N1) 2009 became available at the end of October 2009. Assuming a 2-week period for vaccine administration, case-patients in groups at risk with influenza onset dates after November 30, 2009, could have been vaccinated and would have had time to seroconvert (14 days). Using these criteria, we determined that 4 cases (in 3 adults and 1 child) (13%) were potentially preventable by vaccination; 2 of these patients required escalated care, and 1 patient died. Although 72% of patients received antiviral therapy, similar to 75% in the source cohort (*27*), we observed avoidable delays between recording of respiratory symptoms and start of specific antiviral therapy in some adults and all children. Although under ordinary circumstances the complex clinical picture of such patients might result in delayed or incidental finding of influenza, in a pandemic situation or

during a seasonal epidemic clinicians should be alert to the possibility of influenza. Delays encountered in this series most likely reflect a failure to consider such a diagnosis early. Other reasons are caution or uncertainty in using oseltamivir in patients younger than the drug licensing permits (12 months) in nonpandemic situations, reluctance to empirically instigate antiviral treatment in advance of a confirmed diagnosis of pandemic (H1N1) 2009, lack of confidence about absorption of oseltamivir by nasogastric tube insertion in patients already receiving mechanical ventilation or concerns about potential gastrointestinal side effects.

Nosocomial infections with pandemic (H1N1) 2009 in this case series were associated with high rates of illness and death. This finding highlights the need for adherence to infection control guidelines for staff and visitors (including the need to urge visitors not to visit when they are ill, particularly when providing hands-on care for vulnerable children), staff vaccination, maintenance of clinical suspicion for influenza in areas of high risk, prompt (empirical) antiviral treatment for vulnerable patients in whom influenza is possible or likely, and consideration of postponing nonurgent procedures for hematology patients during periods of known high influenza activity. This report demonstrates that nosocomial transmission is a recurrent problem when the prevalence of influenza is high and the total effect of nosocomial influenza is underestimated by outbreak reports alone.

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Genomic Analysis of Highly Virulent Georgia 2007/1 Isolate of African Swine Fever Virus

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African swine fever is widespread in Africa but has occasionally been introduced into other continents. In June 2007, African swine fever was isolated in the Caucasus Region of the Republic of Georgia and subsequently in neighboring countries (Armenia, Azerbaijan, and 9 states of the Russian Federation). Previous data for sequencing of 3 genes indicated that the Georgia 2007/1 isolate is closely related to isolates of genotype II, which has been identified in Mozambique, Madagascar, and Zambia. We report the complete genomic coding sequence of the Georgia 2007/1 isolate and comparison with other isolates. A genome sequence of 189,344 bp encoding 166 open reading frames (ORFs) was obtained. Phylogeny based on concatenated sequences of 125 conserved ORFs showed that this isolate clustered most closely with the Mkuzi 1979 isolate. Some ORFs clustered differently, suggesting that recombination may have occurred. Results provide a baseline for monitoring genomic changes in this virus.

African swine fever (ASF) is a hemorrhagic fever in domestic pigs that causes serious economic losses and high mortality rates. ASF is currently endemic to many countries in sub-Saharan Africa and the island of Sardinia in Europe and was endemic to Spain and Portugal from 1960 until the mid 1990s. It is still endemic to Madagascar since its introduction in 1998. Sporadic ASF outbreaks have occurred in Brazil, the Caribbean region, the Indian Ocean island of Mauritius, and countries in Europe (*1*). There is no vaccine against ASF, and disease control relies on rapid diagnosis and implementation of quarantine and slaughter policies. African swine fever virus (ASFV) is a large, icosahedral, cytoplasmic, double-stranded DNA virus; it is the only member of the family *Asfaviridae*, although it shares similarities with other virus families in the superfamily of nucleo-cytoplasmic large DNA viruses (*2–4*).

In 2007, a new outbreak of ASF was confirmed in the Republic of Georgia, which is far from the usual geographic virus range in sub-Saharan Africa. Infections were first observed near the Black Sea port of Poti and are thought to have been introduced by improper disposal of waste from shipping. The disease rapidly spread throughout Georgia and was reported in Armenia and in wild boar in Chechnya in the Russian Federation in 2007 and Azerbaijan in 2008. ASF has since spread to 9 regions in the Russian Federation, including 2,000 km to St. Petersburg in October 2009. As of August 10, 2010, there have been 85 outbreaks reported within the Russian Federation, which have led to the deaths of ≈48,000 animals and an estimated cost to the Russian economy during 2009 of US$1 billion (*5*; World Organisation for Animal Health Information Database). There have been several reports of ASFV infection in wild boars in different locations in the Russian Federation, which led to fears that ASF may have become established in the wild boar population. This rapid transboundary spread of ASF emphasizes the serious risk for ASF to pig farming worldwide.

In its natural hosts (warthogs [*Phacochoerus aethiopicus*], bushpigs [*Potamochoerus porcus*], and *Ornithodorous* spp. soft ticks), ASFV causes a persistent but asymptomatic infection. In domestic swine, it causes an acute hemorrhagic infection with mortality rates ≤100%. European wild boars (*Sus scrofa*) are susceptible, and disease signs are similar to those in domestic pigs. The ASFV strain introduced to the Caucasus is highly

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virulent and resulted in a mortality rate of ≈100% during the early stages of the outbreak in Georgia; ≈90,000 animals died or were destroyed (http://web.oie.int/wahis/public.php?page=home). Experimental infections of pigs confirmed that isolates obtained after introduction of ASF into Armenia and the Russian Federation cause acute disease and result in high mortality rates (www.efsa.europa.eu/en/scdocs/scdoc/1556.htm).

Genotyping of ASFV isolates by partial sequencing of the B646L gene that encodes the major capsid protein p72 has identified 22 genotypes (*6*). The Georgia 2007/1 isolate was grouped within genotype II by partial sequencing of the B646L and B602L genes and complete sequencing of the CP204L gene. Genotype II virus has been isolated in Mozambique and Zambia and was also introduced into the Indian Ocean islands of Madagascar (1998) and Mauritius (2007) (*7*).

We analyzed the complete coding region of the genome of the Georgia 2007/1 strain of ASFV, which was isolated after its introduction to Georgia in 2007. This information provides a baseline for comparison with other isolates obtained during the continued spread of ASF in this region and provides information for vaccine and diagnostic test development.

Methods

Viruses and Cells

The Georgia 2007/1 isolate was obtained from tissue samples from pigs submitted to the World Organisation for Animal Health Reference Laboratory at the Institute for Animal Health, Pirbright, UK, on June 4, 2007 (*7*). Primary porcine bone marrow cells cultured in Earle saline media at a concentration of 4×10^6 cells/mL were infected with virus at a multiplicity of infection of 1. Virus-containing cell supernatants were collected 4 days postinfection. Virus-containing cell supernatant was used for purification of virus DNA.

Purification of Virus DNA

Virus supernatant was centrifuged at 118,000 × *g* (SW 32 Ti Rotor; Beckman Coulter, Brea, CA, USA) for 1 h at 4°C. Pelleted virus was resuspended in RSB buffer (10 mmol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA) containing 0.01 M $MgCl_2$ and DNase I (Sigma, St. Louis, MO, USA) (200 μg/mL) and incubated for 1 h at 37°C to digest contaminating cellular DNA. EDTA (50 mmol/L) was then added to inactivate DNase. Virus was then centrifuged through a 20% sucrose RSB cushion at 62,000 × *g* (70.1 Ti Rotor; Beckman Coulter) for 95 min at 4°C. Virus pellets were resuspended in 1 mL of buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA). RNase (40 μg/mL), proteinase K (200 μg/mL), and sodium dodecyl sulfate (1% final concentration) were added, and samples were incubated for 18 h at 37°C. Viral genomic DNA was extracted with phenol and precipitated with ethanol. To remove low molecular weight nucleic acid, viral DNA was further purified by using the Elu-Quick Kit (Whatman, Maidstone, UK) according to the manufacturer's protocol II.

Sequence Determination and Analysis

DNA for sequencing was amplified from 100 ng of purified viral DNA by using the Repli-G Kit (QIAGEN, Valencia, CA, USA). This method uses an isothermal multiple displacement amplification and a processive DNA polymerase capable of replicating ≤100 kbp. The DNA polymerase has a $3' \rightarrow 5'$ exonuclease proofreading activity to maintain high fidelity in the amplified products. Nucleotide sequence of the complete coding regions of the genome of the Georgia 2007/1 isolate was determined by using a Roche (Basel, Switzerland) 454 GS FLX sequencer. Analysis of genome sequences, open reading frames (ORFs), and orthologous protein families were conducted by using Artemis (*8*), Glimmer software (*9*) and programs available at Viral Bioinformatics–Canada (*10*,*11*). ORFs were compared with the related ASFV genome sequences (Mkuzi 1979 isolate, GenBank accession no. AY261362 and Genotype I, Benin 97/1 isolate, GenBank accession no. AM712239) to identify potential frame shifts in the genome that interrupted reading frames. Regions of uncertainty were sequenced by PCR amplification of fragments and Sanger sequencing to confirm the sequence. These uncertainties were located mainly in homopolymer sequences, which have been reported to cause ambiguities during Roche 454 sequencing (*12*,*13*). The GenBank accession no. for the genome sequence is FR682468.

Results

Sequence of Coding Regions

The final assembly of the Georgia 2007/1 isolate produced a genome of 189,344 bp, not including terminal inverted repeats and cross links. This genome is considerably larger than genomes of attenuated ASFV isolates BA71V (GenBank accession no. NC_001659) (170,101 bp) and OURT88/3 (GenBank accession no. AM712240) (171,719 bp). In contrast, genomes available for virulent isolates range from 182,284 bp to 193,886 bp. Dot-plot comparisons of the Georgia 2007/1 genome with other genomes showed that these genomes were collinear, although deletions or insertions were observed in the regions close to the genome termini, particularly in the left genome end as in genomes of other isolates. Most size differences result from gain or loss of members of 5 multigene families (MGF 100, MGF 110, MGF 300, MGF 360, and MGF 530) (*14*–*16*).

Genomic Analysis

Using GATU software (*10*), we identified 166 ORFs (online Technical Appendix, www.cdc.gov/EID/content/17/4/599-Techapp.htm). Of these ORFs, 125 are present in all 11 ASFV isolates sequenced to date. The conserved ORFs include those that encode for structural proteins; proteins involved in virus assembly, enzymes and other factors involved in nucleotide metabolism, DNA replication and repair, mRNA transcription and processing; several involved in regulating host cell pathways; 16 members of the MGFs; and several of unknown function. Of the remaining 42 ORFs, which are not conserved between all 11 ASFV isolates sequenced, 24 are members of the 5 MGFs. The GATU software identified ORFs on the basis of those encoded in reference genomes. To determine if other ORFs may be present, we used Glimmer software (*9*). This analysis identified 189 ORFs, the additional 23, all encoded proteins of <64 aa that lacked sequence similarity with known proteins (online Technical Appendix). Eleven of these ORFs overlapped or were entirely within other larger ORFs. Thus, these ORFs are not likely to represent functional genes.

Genome Comparison of the Georgia 2007/1 Isolate with other ASFV Isolates

To determine the phylogenetic relationship between the Georgia 2007/1 isolate and other ASFV isolates (Table), we compared the concatenated amino acid sequences of proteins encoded by 125 conserved ORFs comprising 40,810 aa (Figure 1). This phylogenic analysis shows that most isolates cluster in 2 main clades. The first group comprises isolates from West Africa and Europe belonging to genotype I. The Mkuzi 1979 and Georgia 2007/1 isolate also fall within this group but are more distantly related to genotype I isolates. The second group comprises other isolates from eastern and southern Africa (Tengani 62, Warthog, Warmbaths, Pretoriskup 96). Two isolates, Malwai lil 20/1 and Kenya 1950, are outliers from these groups.

Comparison of complete genomes shows that most variation is at the left end of the genomes and is caused by presence or absence of different numbers of members of the MGFs (*14*,*15*). Some ORF deletions are observed close to the right genome end, notably between the tissue culture–adapted BA71V isolate and other isolates, including Georgia 2007/1. The isolates showing greatest sequence divergence from the Georgia 2007/1 isolate are the Kenyan 1950 and Malawi Lil20/1 isolates. This sequence divergence is greatest toward the left end of the genome.

A total of 78 ORFs in all 11 isolates share >90% aa identity of proteins encoded. Of these ORFs, only 33 have a confirmed or predicted function. The most conserved proteins include the histone-like structural protein A104R, which is 99%–100% identical in all isolates. The bcl-2-bax homologue (A179L) protein has 98.9%–100% aa identity in all isolates except Kenya 1950 and Benin 97/1, which are 94% identical compared with that of the Georgia 2007/1 isolate and other isolates. Several of the other most conserved proteins encoded are enzymes, including helicase A859L (>95% identity), RNA helicase B962L (>95% identity), prenyltransferase B318L (>95% identity), RNA polymerase 6 C147L (>96% identity), and DNA primase C962R (>97% identity).

The more divergent proteins include several with immunomodulatory functions, such as A238L, which varies in amino acid identity from 58.9% (Malawi Lil20/1) to 81.3% (Mkuzi) compared with Georgia 2007/1. The C-type lectin-like protein EP153R shows 54.9% (Warmbaths) to 79.7% (Warthog) aa identity compared with Georgia 2007/1. The CD2v protein encoded by the EP402R ORF varies from 65.8% (Tengani isolate) to 86.1% (Malawi Lil20/1 genotype VIII). The CD2v and EP153R proteins are transmembrane proteins with reported roles in evading host defenses (*21,22*). The thymidylate kinase (A240L) protein is divergent whereas most other ORFs that encode enzymes are highly conserved. The C84L and E66Lproteins of unknown function also have variable sequences. The virulence-associated protein DP71L (*23*–*25*) is encoded by ASFV isolates as 1 of 2 forms differing in size. Only genotype VIII isolates from Malawi and Zambia and the

Table. Characteristics of African swine fever virus isolates analyzed

Isolate	Country	Host	Year	Virulence	GenBank accession no.	Reference
Georgia 2007/1	Georgia	Domestic pig	2007	High	FR682468	This study
BA71qqV	Spain	Domestic pig	1971	Tissue culture adapted	U18466	(*15*)
Benin 97/1	Benin	Domestic pig	1997	High	AM712239	(*14*)
OURT 88/3	Portugal	Tick	1988	Low	AM712240	(*17*)
Kenya	Kenya	Domestic pig	1950	High	AY261360	(*18*)
Malawi Lil20/1	Malawi	Tick	1983	High	AY261361	(*19*)
Mkuzi	Zululand	Tick	1978	Unknown	AY261362	(*18*)
Pretorisuskop/96/4	South Africa	Tick	1996	High	AY261363	(*18*)
Tengani 62	Malawi	Domestic pig	1962	High	AY261364	(*20*)
Warmbaths	South Africa	Tick	1987	Unknown	AY261365	(*18*)
Warthog	Namibia	Warthog	1980	Unknown	AY261366	(*18*)

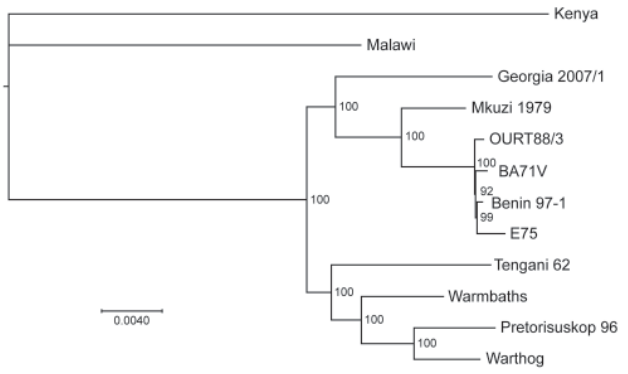

Figure 1. Comparison of the Georgia 2007/1 African swine fever virus (ASFV) isolate genome with those of other ASFV isolates. ASFV phylogeny midpoint was rooted in a neighbor-joining tree on the basis of 125 conserved open reading frame regions (40,810 aa) from 12 taxa. Node values show percentage bootstrap support (n = 1,000). The isolates shown and accession numbers are Kenya AY261360, Malawi Lil20/1 AY261361, Tengani AY261364, Warmbaths AY261365, Pretorisuskop AY261363, Warthog AY261366, Warmbaths AY261365, Mkuzi AY261362, OurT88/3 a.m.712240, BA71V NC_001659, Benin97/1 a.m.712239, and E75 FN557520. Scale bar indicates nucleotide substitutions per site.

Kenya isolates encode the long form. All other isolates, including Georgia 2007/1, encode the short form.

The structural protein P22 (*26*), encoded by the KP177R ORF, is present in only 1 copy near the left genome end of the BA71V isolate; this ORF is present in all the other isolates. However, in the other isolates, there are either 1 or 2 additional ORFs related to KP177R near the right end of the genome. The Georgia 2007/1 isolate contains 1 copy (l10L) of the KP177R-related ORF, close to the right end of the genome. The amino acid identity between the KP177R protein and the related proteins is low, e.g., the 2 proteins share only 42.2% aa identity in the Georgia 2007/1 isolate. Much higher amino acid identity is shared between the proteins encoded by orthologous ORFs from different isolates. For example, the P22 protein is greater than 78% identical across all the genomes analyzed.

Phylogenetic analysis was conducted for proteins encoded by each ORF. Although most proteins showed the same clustering as observed for that of the concatenated conserved ORFs (Figure 1) for several proteins, the Georgia isolate sequence clustered differently. Examples of phylogenetic trees for proteins encoded by 4 ORFs are shown in Figure 2. The A238L and KP177R protein sequences from the Georgia 2007/1 isolate cluster the same as the concatenated conserved 125 proteins, the EP402R protein sequence from the Georgia 2007/1 sequence clusters more closely with the Malawi Lil20/1 and Kenya 1950 isolates, and the EP153R protein sequence from the Georgia 2007/1 isolate clustered more closely with the Warthog isolate. A possible explanation for these observations is that recombination may have occurred. If so, we might expect to find several adjacent ORFs that cluster in the same way and differently from the conserved concatenated ORFs. One such example is observed with the adjacent ORFs l7L (100%), l8L (100%), l9R (100%), and l10L (91.2%) from the Georgia 2007/1 isolate, which encode proteins with the highest amino acid identity with the genotype XIX Warthog isolate. Analysis across the 125 concatenated conserved protein sequences clusters the Georgia 2007/1 isolate more closely with Mkuzi 1979 isolate. However, there is no clear evidence for recent recombination events.

Multigene Families

The presence or absence of some members of MGF 360 and MGF 505/530 families correlates closely with pathogenesis in ASFV, and the complement of these present in the Georgia 2007/1 isolate is as expected for a highly pathogenic isolate. The nonpathogenic isolate OURT 88/3 and the tissue culture–adapted isolate BA71V have deletions of 5 or 6 members, respectively, of MGF 360 and 2 or 1 members, respectively, of MGF 530 (*14*) that are in all other pathogenic isolates sequenced, including the Georgia 2007/1 isolate. Deletion of these members of the MGF 360 and MGF 530 families from the genome of the pathogenic Pretoriuskop 96/4 isolate dramatically reduced virus virulence in domestic pigs (*28*).

The Georgia 2007/1 isolate has 37 members of the different MGFs (online Technical Appendix). In addition, the Georgia 2007/1 isolate only has 1 member of MGF 100 (MGF100–1R) in comparison with other genomes, which have 2 or 3 MGF 100 members. The Georgia 2007/1 genome contains 12 of the 14 known members of MGF 110, including a fusion of MGF 110 5L and 6L (MGF 110–5L/6L). The fusion of these 2 ORFs was confirmed by Sanger sequencing to ensure that it was not a sequencing error. The Georgia 2007/1 isolate has only 2 of the 4 members of MGF 300 compared with a minimum of 3 of 4 found in all other genomes. This isolate contains 15 members of MGF 360; the number present in the other genomes varies from a minimum of 11 or 12 in the BA71V and OURT88/3 isolates, respectively, to 18 in the Kenyan isolate of the 22 MGF genes identified. MGF 505/530 appears to be closely conserved across most genomes. The Georgian2007/1 isolate and 6 other isolates (Benin97/1, Mkuzi, Pretorisuskop, Tengani, Warmbaths, and Warthog) contain 10 of the 11 MGF 505/530 members identified. Nonpathogenic isolates OURT88/3 and BA71V lack 2 (MGF 505/530–1R and –2R) or 1 (MGF 505/530–1R) of the MGF 505/530 ORFs, respectively. Further investigation into the role of individual members of the 5 MGFs on interferon response is ongoing.

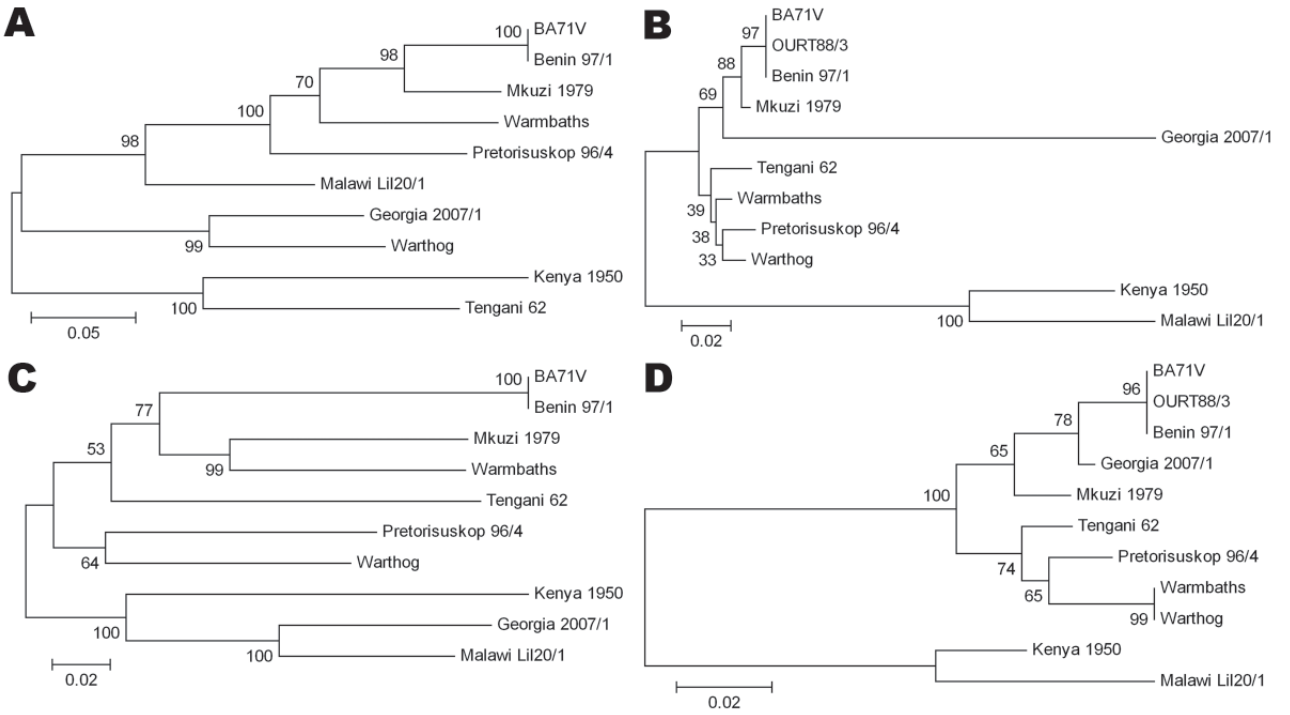

Figure 2. Phylogenetic trees of 4 of the most divergent African swine fever virus proteins. A) C-type lectin EP153R, B) A238L, C) CD2-like protein EP402R, D) structural protein K177R (P22). Evolutionary history was inferred by using the neighbor-joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the proteins analyzed. Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated proteins clustered in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were 224 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (*27*). Scale bars indicate amino acid substitutions per site.

Discussion

The continuing outbreak of ASF in the Caucasus region is caused by a highly virulent strain of ASFV that belongs to genotype II (*7*). Comparison of the nucleotide sequence of the genome of the Georgian 2007/1 isolate with other isolates indicated that it is most closely related to isolate Mkuzi 1979 (Figure 1). The Mkuzi 1979 isolate was obtained from a tick isolate in Zululand near Mozambique where genotype II isolates have been found in domestic pigs. Phylogenetic analysis of concatenated protein sequences from 125 conserved ORFs results in clustering of the Georgia 2007/1 and Mkuzi 1979 isolates with genotype I isolates, although more divergent than other members of this group from West Africa and Europe (Benin 97/1, OURT88/3, BA71V and E75) and other isolates from eastern and southern Africa, including Tengani, 62, Warthog, Warmbaths, and Pretorisuskop 96/4. The eastern Africa isolates Malawi Lil20/1 and Kenya 1950 form a separate and more distantly related cluster.

Analysis of the phylogeny of individual proteins (Figure 2) does not always match the clustering observed by comparison of concatenated conserved ORFs (Figure 1). A possible explanation for this observation is that recombination events have occurred. These observations indicate that caution should be used when inferring phylogenetic relationships between ASFV isolates based on a small number of genes. ASFV isolates have been grouped into 22 genotypes by partial sequencing of the ORF encoding the p72 major capsid protein B646L. However, analysis of the protein sequence encoded by this ORF does not reflect the phylogeny, as indicated by analysis of the concatenated conserved ORFs and of other individual ORFs. Complete genome sequence analysis provides the most information; as viral genome analysis and sequencing becomes more routine, this procedure will become the method of choice. In the short term, targeted sequence analysis of several ORFs, including those that more closely cluster with that of the concatenated conserved ORF sequences, will provide a more accurate estimate of phylogenetic relationships rather than analysis of 1 ORF such as B646L.

Comparison of the rates of synonomous versus nonsynonomous substitutions across ASFV genes identified 14 or 18 genes that are undergoing positive selection (*29*).

These genes included 2 of the proteins (CD2v and EP153R) that we identified as being most divergent at the amino acid level.

Determination of the sequence of the ASFV isolate that was introduced into the Caucasus region provides a benchmark to which other isolates from this epidemic can be compared. This finding may enable sequence changes to be related to any changes in phenotype of the virus. In addition, detailed knowledge of the sequence will facilitate research on vaccine development by enabling the genes encoded to be expressed and assayed for their ability to confer protection in pigs. It will also facilitate the design of rationally attenuated vaccines by sequential deletion of genes involved in immune evasion and virulence.

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Dr Chapman is a postdoctoral researcher at the Institute for Animal Health, Pirbright Laboratory, Woking, UK. His research interests are development of vaccines and pen-side diagnostic systems for ASF and the entry mechanisms of ASFV.

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Diarrheagenic Pathogens in Polymicrobial Infections

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During systematic active surveillance of the causes of diarrhea in patients admitted to the Infectious Diseases and Beliaghata General Hospital in Kolkata, India, we looked for 26 known gastrointestinal pathogens in fecal samples from 2,748 patients. Samples from about one-third (29%) of the patients contained multiple pathogens. Polymicrobial infections frequently contained *Vibrio cholerae* O1 and rotavirus. When these agents were present, some co-infecting agents were found significantly less often ($p = 10^{-5}$ to 10^{-33}), some were detected significantly more often ($p = 10^{-5}$ to 10^{-26}), and others were detected equally as often as when *V. cholerae* O1 or rotavirus was absent. When data were stratified by patient age and season, many nonrandom associations remained statistically significant. The causes and effects of these nonrandom associations remain unknown.

The estimated worldwide death rate from diarrheal diseases is ≈2.2 million deaths per year (*1*). Diarrheal infections may be caused by an array of bacterial, viral, or parasitic pathogens. Some cases have 1 single defined cause, others do not have any defined cause, and a substantial number (one third) are caused by multiple pathogens (*2*). Because each known diarrheal pathogen fulfills Koch's postulates and is capable of being the sole etiologic agent causing disease, multiple pathogens are not essential for causing disease. How additional pathogens cause and contribute to the disease process is unknown. The source of the multiple pathogens in a patient could simply result from multiple pathogens in an urban environment of crowded, impoverished conditions. If the various pathogens occurred independently in cases of disease, then each pathogen in a polymicrobial infection would be expected to occur in proportion to its presence in all patients with severe diarrhea.

In Kolkata, India, a megacity with a population >10 million, many persons live in crowded urban slums. Medical attention is available at the Infectious Disease and Beliaghata General Hospital, which serves the population of Kolkata. To determine the extent of disease caused by various bacterial, viral, and parasitic pathogens of the gastrointestinal tract, the National Institute of Cholera and Enteric Disease is conducting a systematic survey of patients hospitalized for diarrhea at this hospital. Analyses conducted after 2 years of data collection revealed that approximately one-third (29%) of patients had polymicrobial infections (*2*); an earlier report from that ongoing study indicated that the 3 parasites detected most often (in 73% of patients with polymicrobial infections) were *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium* spp. (*3*). We used data from the same ongoing survey to identify gastrointestinal tract pathogens in the feces of patients with severe diarrhea and to examine the relationships between co-infections of *Vibro cholerae* O1 and rotavirus with other bacterial, viral, and parasitic pathogens.

Methods

Details of sample collection and microbiological analyses have been published (*2*). The protocol has been approved by the Institutional Review Board at the National Institute of Cholera and Enteric Disease. Briefly, fecal specimens were collected systematically from patients entering the hospital from November 2007 through

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February 2010. Of note, the previous study analyzed data through October 2009; however, the systematic sampling is still ongoing. The specimens were collected from every fifth patient with diarrhea on 2 randomly selected days each week. Only patients with diarrhea (defined by World Health Organization guidelines as passage of ≥3 loose or liquid stools per day or more frequently than is normal for the person) were eligible for inclusion in the study. Samples were collected from an average of 5.6% of eligible patients.

Each patient contributed 1 sample, and each sample was tested for all 26 common diarrheagenic pathogens. Standard microbiological techniques were used to examine the samples. Samples were collected in McCartney bottles (using sterile catheters or rectal swabs) containing Cary-Blair medium and examined (within 2 hours of collection) for bacterial, viral, and parasitic pathogens by a combination of conventional, immunologic, and molecular methods. The bacterial pathogens (*V. cholerae*, *V. parahaemolyticus*, *V. fluvialis*, *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella* spp., *Shigella* spp., and diarrheagenic *Escherichia coli*) were isolated from appropriate selective media and identified by standard biochemical tests. Species and subtypes were confirmed by serotyping (for *V. parahaemolyticus, Shigella* spp., and *Salmonella* spp.) with commercially available antiserum (Denka Seiken, Tokyo, Japan; BioRad, Marnes-la-Coquette, France) and by PCR (for *V. cholerae* [*4*], *V. fluvialis* [*5*], enterotoxigenic *E. coli* [ETEC, including heat-labile and heat-stable enterotoxin producers], enteropathogenic *E. coli* [EPEC, typical and atypical], enteroaggregative *E. coli* [EAEC] [*6*], enteroinvasive *E. coli*, and Shiga toxin–producing *E. coli* [*7*]). Rotavirus was detected by polyacrylamide gel electrophoresis and silver staining (*8*). Noroviruses (groups I [NVG1] and II [NVG2]), sapovirus, and astrovirus were detected by reverse transcription–PCR with random primers for reverse transcription and specific primers for PCR (*9*). Adenoviruses were detected by the commercially available RotaAdeno VIKIA Kit (bioMérieux, Marcy l'Etoile, France). All samples were screened by using a highly sensitive antigen capture ELISA (TechLab, Inc., Blacksburg, VA, USA) of *G. lamblia*, *Cryptosporidium parvum, E. histolytica*, and *Blastocystis hominis.*

To test for possible associations, we used the Fisher exact test to compare pairs of pathogens (1, both, or neither) with an independent assortment based on the overall frequency with which pathogens were detected. To establish criteria for statistical significance, we calculated p values, odds ratios (ORs), and 95% confidence intervals (CIs). Additional covariates were collected and examined for confounding and interaction. These included patient age, gender, residence, and religion and season of infection. Seasons were defined as summer (March–June), monsoon (July–October), and winter (November–February). All analyses were conducted by using SAS version 9.2 (SAS Institute, Cary, NC, USA).

Results

Fecal samples were submitted from 2,748 patients. Patient demographic characteristics are listed in Table 1. A large proportion (44%) of patients were 15–45 years of age, ≈13% were ≤1 year of age, 80% resided in urban areas, 74% were Hindu, and 25% were Muslim. The following pathogens were detected in at least 1 sample: adenovirus, *Aeromonas* spp., astrovirus, *B. hominis*, *C. jejuni*, *C. parvum,* EAEC, EPEC, ETEC, *E. histolytica*, *G. lamblia*, NVG1, NVG2, rotavirus, *Salmonella* spp., sapovirus, *Shigella* spp., *V. cholerae* O1, *V. cholerae* O139, *V. cholerae* non-O1, *V. cholerae* non-O139, *V. parahaemolyticus*, and *V. fluvialis*. No pathogens were

Table 1. Characteristics of 2,748 patients hospitalized with diarrhea, Kolkata, India, November 2007–February 2010*

Characteristic	Total, no. (%)
No. pathogens	
0	766 (27.9)
≥1	1,982 (72.1)
1	1,169 (42.5)
2	589 (21.4)
3	165 (6.0)
4	44 (1.6)
5	10 (0.4)
6	5 (0.2)
Age group, y*	
≤1	360 (13.1)
>1–2	233 (8.5)
>2–5	177 (6.4)
>5 –15	243 (8.8)
>15 –45	1,210 (44.0)
>45	525 (19.1)
Gender	
M	1,482 (53.9)
F	1,266 (46.1)
Residence	
Urban	2,226 (81.0)
Rural	522 (19.0)
Religion	
Hindu	2,043 (74.3)
Muslim	698 (25.4)
Christian	5 (0.2)
Other	2 (0.1)
Season	
Nov–Feb	890 (32.4)
Mar–Jun	837 (30.5)
Jul–Oct	1,021 (37.1)
Feces	
Watery	2,080 (75.7)
Loose	561 (20.4)
Bloody	21 (0.8)
Mucoid	15 (0.5)
Bloody and mucoid	71 (92.6)

*Mean ± SD patient age 26 ± 22 y.

detected in 766 (28%) of the 2,748 samples (Table 1), but test results were positive for the other 72%. One pathogen was found for 1,169 (43%) samples and multiple pathogens for 813 (29%) (Table 1). The 2 most commonly detected pathogens were *V. cholerae* O1 and rotavirus, which were found in 24% and 22% of samples, respectively.

V. cholerae O1 was detected in 661 samples. *V. cholerae* was the sole pathogen in 379 samples; however, it was isolated along with another diarrheagenic pathogen from 282 samples. The co-infection of *V. cholerae* and rotavirus was highly significant ($p = 1.12 \times 10^{-33}$). Co-infection with *V. cholerae* and rotavirus was ≈5-fold less likely (OR 0.18, 95% CI 0.13–0.25; Figure, panel A) to occur among those with than among those without *V. cholerae* infection. A negative association might be expected if a case of severe diarrhea caused by any given pathogen excluded other pathogens. Consistent with this expectation, the presence of *C. parvum*, adenovirus, *Shigella* spp., ETEC, and *V. parahaemolyticus* was decreased significantly ($p = 7.87 \times 10^{-5}$ to 1.32×10^{-9}) and was 12.5-fold (with *V. parahaemolyticus,* OR 0.1, 95% CI 0.02–0.33) to 2.44-fold (with *C. parvum*) less likely to occur among those with than among those without *V. cholerae* infection. However, antithetically, the rate of *G. lamblia* co-infection was significantly higher among *V. cholerae* O1–positive than among *V. cholerae* O1–negative fecal samples (OR 1.71, 95% CI 1.32–2.21). A significant difference in infection rates among those with and without *V. cholerae* O1 infection was not found for EAEC, *C. jejuni*, *V. fluvialis*, *E. histolytica*, astrovirus, NVGII, and EPEC. Tests for association were not performed for *Salmonella* spp., NVGI, *Aeromonas* spp., *B. hominis*, *C. coli*, sapovirus, *V. cholerae* non-O1, *V. cholerae* non-O139, and *V. cholerae* O139 because the low number of patients infected with those pathogens resulted in insufficient power.

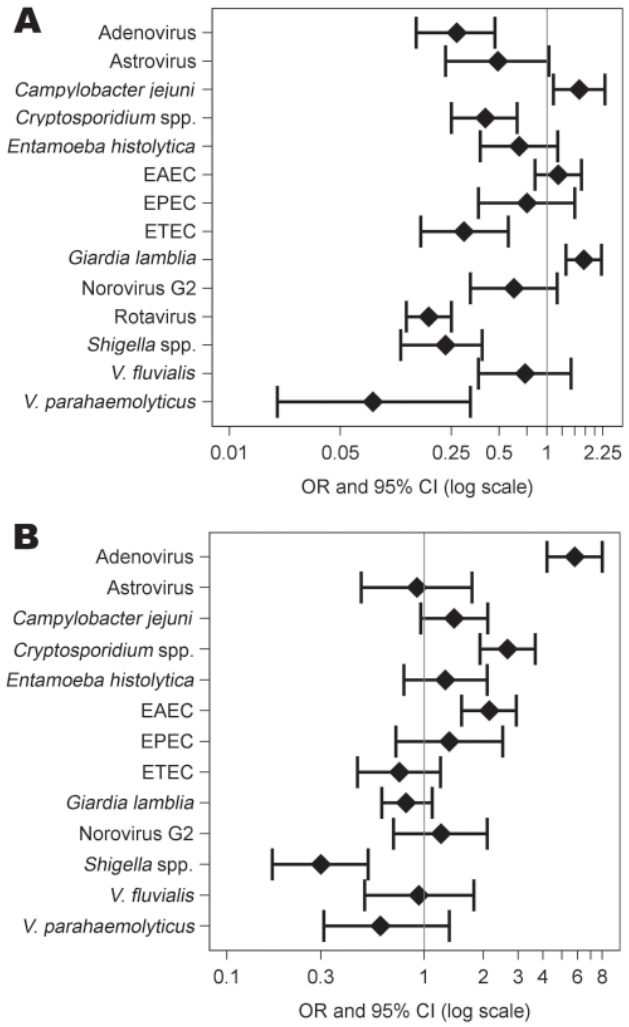

Figure. Odds ratios (ORs) showing odds of A) *Vibrio cholerae* or B) rotavirus co-occurring with various other pathogens relative to the odds of *V. cholerae* or rotavirus co-occurring independently with various other pathogens at the frequency with which each is present in the entire sample. This standard forest plot indicates the best estimate and the 95% confidence intervals (CIs) for each co-occurring organism. EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *E. coli;* ETEC, enterotoxigenic *E. coli.*

Rotavirus was detected in 594 of the fecal samples and was the sole pathogen found in 253 of them. Rotavirus and at least 1 other gastrointestinal pathogen were found in 341 samples; 119 samples were co-infected with rotavirus and ≥2 other pathogens. When the effect of rotavirus co-infection with other pathogens was tested (Figure, panel B), *Shigella* spp. were significantly less likely to be found in samples with rotavirus than in samples without rotavirus (OR 0.30, 95% CI 0.17–0.52). In contrast, EAEC, *Cryptosporidium* spp., and adenovirus were significantly increased in samples with rotavirus ($p = 6.15 \times 10^{-6}$ to 1.61×10^{-26}; ORs 2.14–5.80. A significant effect was not observed for *G. lamblia*, *C. jejuni*, EPEC, ETEC, *V. parahaemolyticus*, *V. fluvialis*, *E. haemolyticus*, astrovirus, and NVGII. Tests for association were not performed for *Salmonella* spp., NVG1, *Aeromonas* spp., *B. hominis*, *C. coli*, sapovirus, *V. cholerae* non-O1, *V. cholerae* non-O139, and *V. cholerae* O139 because the low number of patients infected with those pathogens resulted in insufficient power. Analysis of samples from patients infected simultaneously with *G. lamblia*, *V. cholerae*, and rotavirus (n = 41) revealed that the frequency of co-infection with *G. lamblia* was not significantly affected by co-infection with *V. cholerae* O1 and rotavirus ($p = 0.08$).

Analysis of covariates indicated that gender, religion, and residence largely had no effect on the associations between pathogens; however, in some instances, age and season were identified as confounders or effect modifiers (Table 2). To examine the effect of these covariates, we stratified the data by age and season and found that many associations remained significant (online Appendix Table,

Table 2. Effect of covariates on gastrointestinal pathogen associations*

Pathogens	Covariate					
	Age	Season	Gender	Residence	Religion	After adjusting for effects
Vibrio cholerae/rotavirus	Confounder	Interaction	No effect	No effect	No effect	Significant except for age strata 5–15 y
V. cholerae/adenovirus	Confounder	No effect	No effect	No effect	No effect	Significant when regression adjusted for age and season (OR 0.36, 95% CI 0.21–0.64)
V. cholerae/*Cryptosporidium* spp.	Confounder	Confounder	Interaction	No effect	No effect	Significant for female, not male patients; small stratified cell sizes
V. cholerae/*Giardia lamblia*	Interaction	Confounder	No effect	Interaction	No effect	Significant for some age categories
V. cholerae/*Shigella* spp.	Interaction	No effect	No effect	No effect	No effect	Significant for ages >2 y and all seasons
V. cholerae/ETEC	No effect	Interaction	No effect	No effect	No effect	Significant for summer and monsoon seasons
Rotavirus/adenovirus	Interaction	Interaction	No effect	No effect	No effect	Significant for all seasons and all age strata except <1 y
Rotavirus/*Cryptosporidium* spp.	Confounder	Confounder	No effect	No effect	No effect	Significant when regression adjusted for age and season (OR 1.64, 95% CI 1.11–2.41)
Rotavirus/EAEC	Confounder	No effect	No effect	No effect	Interaction	Not significant when regression adjusted for age and season (OR 1.38, 95% CI 0.94–2.01); significant for age strata 2–5 y; all seasons significant
Rotavirus/*Shigella* spp.	Confounder	Confounder	No effect	No effect	No effect	Significant when regression adjusted for age and season (OR 0.24, 95% CI 0.14–0.44)

*Interaction identified using Breslow-Day test for homogeneity, p<0.05. Considered statistically significant at p<0.05. OR, odds ratio; CI, confidence interval; ETEC, enterotoxigenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*.

www.cdc.gov/EID/content/17/4/606-appT.htm). Associations between rotavirus and adenovirus remained significant for all age and season strata except among children ≤1 year of age. After adjusting for age and season by using logistic regression models, we found that co-infection with rotavirus and *Cryptosporidium* spp. and co-infection with rotavirus and *Shigella* spp. remained significant (Table 2). The negative association between *V. cholerae* and adenovirus remained significant after adjustment by logistic regression for age and season (OR 0.36; 95% CI 0.21–0.64); associations between *V. cholerae* and many other pathogens remained significant within specific strata of age and season. Assessing the effect of covariates was limited in some instances because of small cell sizes. For this reason, we did not include stratified results for co-infection with *V. cholerae* and *V. parahaemolyticus* in the online Appendix Table.

Discussion

Our analyses revealed that co-occurrence of gastrointestinal pathogens in feces of patients with polymicrobial infections and severe diarrhea necessitating hospitalization was not in proportion to the pathogens' presence in all patients with diarrhea. Tests for association were performed with *V. cholerae* O1 and rotavirus because they were the most commonly detected pathogens and, hence, had the greatest power to detect an association with the other pathogens. Some combinations of pathogens occurred less frequently than expected (e.g., *V. cholerae* and rotavirus [OR 0.18, 95% CI 0.13–0.25]); some combinations appeared more frequently than expected (e.g., rotavirus and adenovirus [OR = 5.8, 95% CI 4.20–7.99]), and some combinations occurred at the same frequency whether with or without *V. cholerae* or rotavirus. After adjustment for age and season, these variables often acted as confounders or effect modifiers, but in general the associations remained significant. However, many of the stratified analyses had small numbers for comparison.

V. cholerae O1 exhibited a positive association with only *G. lamblia*, suggesting that something may be unique about the co-occurrence of those 2 gastrointestinal pathogens. In support of that idea are 1) a report that co-infection with *G. lamblia* and *V. cholerae* results in *G. lamblia* being present in trophozoite form rather than in the cyst form found in feces of control patients (*10*), and 2) a previous finding that *G. lamblia* trophozoites can bind cholera toxin (*11*). Alternatively, each is a pathogen with substantial environmental reservoirs, and the positive association may simply represent acquisition of both pathogens from the same environmental source.

Phylogenetic relatedness alone does not explain the apparent competitive inhibition or negative association that we found between *V. cholerae* and other pathogens. For example, although the closely related *V. cholerae* O1 and *V. parahaemolyticus* exhibited a 10-fold negative association, *V. fluvialis*, which is phylogenetically only slightly farther from *V. cholerae* O1 than is *V. parahaemolyticus*, did not show any inhibition in the presence of *V. cholerae*. Also, although 2 members of the family *Enterobacteriaceae* (*Shigella* spp. and ETEC) were found less frequently than expected in combination with *V. cholerae* O1, 2 other members of that family (EPEC and EAEC) occurred in the expected proportion in samples from patients with mixed *V. cholerae* O1 infections.

Rotavirus had multiple strong positive associations (ORs >2) with the other gastrointestinal pathogens detected in the fecal samples, even after considering age and season. The positive association of rotavirus, an RNA virus that affects cells covered in microvilli, and adenovirus, a DNA virus that affects cells that are dividing to generate new cells with microvilli, may represent an interaction between pathogens to cause more severe diarrhea (in our sample all patients were hospitalized) in patients 15–45 years of age (OR 10.81, 95% CI 5.63–20.78) or a way to escape or circumvent immunity from previous exposures. In a previous study, Koh et al. (*12*) found that among children in Korea with virus-caused diarrhea, adenoviruses occurred preferentially in the presence of rotavirus and that rotavirus and norovirus, although most common, occurred in polymicrobial infections in proportion to their numbers in the samples. Both observations are consistent with the results of our study. Furthermore, Bilenko et al. (*13*) observed that among Bedouins, *G. lamblia* was frequently found in polymicrobial infections and, when present with rotavirus, produced less severe diarrhea than rotavirus alone. In addition, Souza et al. (*14*) found that among young children in São Paulo, those with rotaviral and bacterial co-infections were more likely to have severe diarrhea than were children infected with either pathogen alone. However, the study had insufficient power to examine differences among bacterial pathogens, as did the rest of the studies of rotavirus infections mentioned in a recent review (*15*).

The presence of multiple pathogens in one third of patients with diarrhea has potential implications for treatment and raises several questions. Do cases of diarrhea caused by *V. cholerae* or rotavirus and a second pathogen differ from those caused by *V. cholerae* or rotavirus alone? Does 1 pathogen lead the way for another to successfully infect a person? Do the pathogens behave synergistically to escape immunologic detection? Because the cross-sectional nature of our study did not enable us to investigate the temporal sequence of pathogen infection, future research is needed to provide more evidence concerning the causal pathway(s). Also, the clinical significance of our findings must be more rigorously evaluated by studies that include infected patients and controls. A more substantive investigation into how age and season might affect polymicrobial infections should also be conducted.

The results of our current study indicate that associations can occur between some pathogens affecting the human gastrointestinal tract. The observation of selective positive associations among some gastrointestinal pathogens raises the question of how they interact in vivo; e.g., is the critical factor a modification of gastrointestinal tract microflora? Understanding the association(s) among various co-infecting pathogens may help direct the development of treatment strategies.

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Bordetella petrii Infection with Long-lasting Persistence in Human

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We report the repeated isolation of *Bordetella petrii* in the sputum of a 79-year-old female patient with diffuse bronchiectasis and persistence of the bacterium for >1 year. The patient was first hospitalized due to dyspnea, which developed into severe cough with purulent sputum that yielded *B. petrii* on culture. After this first episode, the patient was hospitalized an additional 4 times with bronchorrhea symptoms. The isolates collected were analyzed by using biochemical, genotypic, and proteomic tools. Expression of specific proteins was analyzed by using serum samples from the patient. The *B. petrii* isolates were compared with other *B. petrii* isolates collected from humans or the environment and with isolates of *B. pertussis, B. parapertussis, B. bronchiseptica,* and *B. holmesii,* obtained from human respiratory tract infections. Our observations indicate that *B. petrii* can persist in persons with chronic pulmonary obstructive disease as has been previously demonstrated for *B. bronchiseptica*.

The genus *Bordetella* comprises 9 species; all, except *B. petrii,* are obligatorily associated with host organisms (*1*). The first isolations of *B. ansorpii* were from a cyst (*2*) and from a blood sample (*3*), whereas *B. trematum* has been isolated from infected ears and from wounds in humans. The reservoir and the pathogenic role of these 2 species remain unknown (*4*). *B. pertussis,* a strictly human pathogen, and *B. parapertussis,* a pathogen in both humans and sheep, are agents of whooping cough (*5*). *B. avium* and *B. hinzii* caused respiratory infections in birds and poultry and have also been reported to cause infections in humans (*6–9*). The latter 4 *Bordetella* spp. are usually described as extracellular bacteria that secrete adhesins and toxins adapted to their hosts (*10*).

However, 2 other *Bordetella* species, *B. bronchiseptica* and *B. holmesii*, behave differently and are able to persist inside their hosts. *B. bronchiseptica* is a respiratory pathogen which may cause acute or chronic bronchopneumonia and is found in many animals, including dogs, cats, pigs, and rabbits, as well as humans (*11*,*12*). *B. holmesii*, originally described as Centers for Disease Control and Prevenion nonoxidizer group 2 (NO-2), has been isolated from the blood cultures of young adults, mostly with underlying disorders or from sputum (*13–15*). The reservoir of this bacterium is unknown. However, *B. petrii* has also been isolated from patients with cystic fibrosis (*16–19*). Unlike the "classical" pathogenic species, *B. pertussis* and *B. parapertussis*, *B. petrii, B. holmesii,* and *B. bronchiseptica,* have the ability to acquire or exchange genomic regions (*20*,*21*). *B. petrii* possesses the largest number of huge genomic islands collectively known as integrative and conjugative elements (*22*,*23*). Several determinants of virulence expressed by *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica,* such as filamentous hemagglutinin (FHA), pertactin (PRN), and fimbriae (Fim2 and Fim3), and toxins such as pertussis toxin (PT) and adenylate cyclise-hemolysin (AC-Hly) were not detected in *B. petrii,* except for an FHA-related adhesin with low similarity (*22*). In this study, we describe an immunocompetent adult with predisposing factors (chronic obstructive respiratory disease and local corticotherapy) who acuired an acute *B. petrii* infection that had long-lasting persistence.

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Materials and Methods

Case History

A 79-year-old nonfebrile woman was hospitalized in October 2007 with dyspnea, which had progressed

over 1 week to severe coughing with abundant purulent and hemoptoic sputum, although the patient had been treated with respiratory physiotherapy and corticosteroid aerosols at home (which was in poor hygienic condition). C-reactive protein level was only moderately elevated (27 mg/mL, reference <10 mg/mL). This patient had experienced pulmonary tuberculosis 33 years earlier and had been successfully treated. For 30 years, she had diffuse bronchiectasis, which had required a right middle lobectomy 23 years previously. She had a myocardial infarction 6 years ago. The patient also had severe chronic hyponatremia, diagnosed as Schwartz-Bartter syndrome, which may have been related to a chronic respiratory deficiency. At admission, a purulent sputum sample was taken (class 5 of Bartlett-Murray and Washington criteria) (*24*) yielded a monomicrobial culture (10^8 CFU/mL) of *B. petrii*. After receiving empirical treatment with amoxicillin-clavulanate, the patient improved slowly and was discharged after 2 weeks. Over the next 12 months, the patient returned with bronchorrhea symptoms in November and December 2007 and in May and November 2008. Each time, *B. petrii* was isolated from purulent sputum specimens in either pure or mixed cultures (in the second sputum culture, *B. petrii* 10^8 CFU/mL and *Citrobacter freundii* 10^4 CFU/mL; in the third sputum culture, *B. petrii* 10^8 CFU/mL and *C. freundii* 10^7 CFU/mL; in the fourth culture, *B. petrii* 10^7CFU/mL pure culture; in the fifth culture, *B. petrii* 10^7 CFU/mL and *Haemophilus parainfluenzae* 10^7 CFU/mL; and in the last sputum culture, *B. petrii* 10^7 CFU/mL pure culture). These episodes did not require hospitalization, and the patient's symptoms were empirically treated with amoxicillin and clavulanate. However, during these repetitive episodes, the patient did not have any general symptoms of sepsis (no fever nor elevated C-reactive protein level). The reactivation of tuberculosis and infection with other mycobacteria were excluded by 3 microscopic examinations and liquid and solid cultures. The patient died in January 2009 from deep electrolytic disorders related to Schwartz-Bartter syndrome.

Bacterial Isolation, Identification, DNA Extraction, and Growth Conditions

Sputum samples (*25*) were plated onto the following agar plates: chocolate PolyViteX agar for bacterial numeration, bromo-cresol purple (BCP) agar plates, selective *Haemophilus* (chocolate bacitracin) agar, Columbia agar with nalidixic acid and 5% sheep blood (all from bioMérieux, Marcy-l'Etoile, France), and CHROMagar Candida (BBL, Becton, Dickinson and Company, Le Pont de Claix, France). Plates were incubated at 37°C for 24 h and for 48 h in a humidified atmosphere with 9% CO_2. For each sample, 10^6–10^8 CFU/mL were observed and characterized. At 48 h, small colonies of a gram-negative bacterium were detected on the first 3 media. Routine identification was performed by a Gram-Negative Identification card on a VITEK 2 automate (bioMérieux) and manually by using API 20NE, API 32GN (bioMérieux), and RapID NH (Remel, Lenexa, KS, USA) strips in accordance with the manufacturer's instructions.

Six isolates were collected sequentially from the patient over 13 months. We chose to analyze the first (October 2007), middle (May 2008), and final (November 2008) isolates from the patient and compared them with the other *B. petrii* isolates. The *Bordetella* reference strain and clinical isolates used in this study are listed in Table 1. Bacterial suspensions were prepared from bacteria grown on Bordet-Gengou agar, supplemented with 15% defibrinated blood (BGA, Difco, Detroit, MI, USA) for 24 h at 36°C, which were then resuspended in saline at 1.8×10^{10} CFU/mL.

Sequencing of the 16S rRNA, *risA*, and *ompA* Genes

DNA from the selected isolates was extracted by using a DNeasy blood and tissue kit (QIAGEN, Courtaboeuf, France). DNA amplification of the 16S rRNA gene was performed as previously described (*29*,*30*). Amplification and sequencing of the genes for the *Bordetella* outer membrane protein A (*ompA*) and the response regulator (*risA*) was performed by using the method described by von Wintzingerode et al. (*1*) with a few modifications. To optimize PCR amplification conditions, we designed primers for the *ompA* gene (ompA3e: 5′-CTC CTC CAA

Table 1. Reference strains and isolates of *Bordetella* spp. isolates used in this study, October 2007–November 2008

Isolate	Species	Year collected	Origin	Reference
FR3799	*B. petrii*	2007	Human	This study
FR3891	*B. petrii*	2008	Human	This study
FR 3996	*B. petrii*	2008	Human	This study
FR3497	*B. petrii*	1995	Human	(*17*)
KMBW	*B. petrii*	Unknown	Environment	(*1*)
CIP 8132 (Tohama)	*B. pertussis*	1985	Human	(*26*)
Bpp12822	*B. parapertussis*	1993	Human	(*26*)
Bbs RB50	*B. bronchiseptica*	Unknown	Rabbit	(*27*)
Bho1	*B. holmesii*	2007	Human	(*28*)

ATT CGC TCT GGC-3′ and ompA4b: 5′-GCA GTT CGC CCT TGC CTT-3′) and *risA* gene (RisA1c: 5′-AAA ACA CCA ATC CCA TCC GC-3′ and RisA2d: 5′-ACA GGT TGA GCA CAT AGG GC-3′).

The nucleotide sequences of 16S rRNA, *risA*, and *ompA* genes from the 3 isolates of the patient (FR3799, FR3891, FR3996) and from the other isolate of human origin (FR3497) have been submitted to the EMBL Nucleotide Sequence Database under the respective accession numbers FN691469/FN691470/FN691471/FN691472; FR669151/FR669152/FR669153/FR669154; and FR669155/FR669156/FR669157/FR669158. For comparative sequence analysis of the 3 different genes, the software, clustalW was used (*31*).

Matrix-assisted Laser Desorption and Ionization Time-of-Flight (MALDI-TOF) Identification

A MALDI-TOF Axima Assurance (Shimadzu-Biotech Corp., Kyoto, Japan) was used. A positive ion mode, liner mode of detection was running, with a laser frequency of 50 Hz and a mass range window of 2,000–30,000 kDa. The sample was prepared, in duplicate, by a direct deposit of a colony fraction on a target plate, and addition of 1 μL of matrix (acide α-4-cyano-4-hydroxycinnamique, AnagnosTec, Potsdam-Golm, Germany), and drying at ambient temperature. Controls and calibration were done with *Escherichia coli* CCGU 10979 (Culture Collection Göteborg University, Göteborg, Germany). One hundred spectra were obtained, with Launchpad version 2.8 software for spectrum acquisition (Shimadzu-Biotech Corp). Spectra were analyzed with Saramis software, version 3.3.2 (AnagnosTec).

Antimicrobial Drug Susceptibility Testing

MICs were determined by using Etest strips (AB Biodisk, Solna, Sweden; bioMérieux) in accordance with the manufacturer's recommendations (EAS 004 2007–6), with 0.5 McFarland inoculum on Mueller-Hinton medium, supplemented with 15% horse blood in an atmosphere of 9% CO_2 at 37°C (*32*,*33*). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. We tested the following: penicillin, amoxicillin, piperacillin, piperacillin and tazobactam, cefotaxime, ceftazidime, ertapenem, imipenem, meropenem, doripenem, gentamicin, tobramycin, amikacin, levofloxacin, ciprofloxacin, moxifloxacin, minocycline, tygecycline, cotrimoxazole, fosfomycin, quinupristin and dalfopristin, rifampin, daptomycin, linezolid, clindamycin, and fucidic acid.

Additional Tests

Pulsed-field gel electrophoresis (PFGE) analysis was performed as described by Caro et al. (*34*). Western blot analyses were performed as previously described (*35*). Serum specimens used included polyclonal specific murine serum specimens (anti-FHA, PRN, PT, AC-Hly) (*35*), the serum collected 7 months after the hospitalization of the patient infected with *B. petrii,* and 2 pools of serum samples from patients infected with *B. pertussis* and *B. bronchiseptica,* respectively.

Fimbrial protein expression was detected by agglutination with monoclonal anti-Fim2 and anti–Fim-3 antibodies. Cytotoxicity of bacteria to murine alveolar macrophage J774-*A1* cells was measured as previously described by Bassinet et al. (*36*).

Results

The bacteria isolated from the patient grew slowly on the chocolate, *Haemophilus* chocolate, and BCP agar plates. On routine media, the bacteria were irregular gram negative coccobacillus, nonmotile, and strictly aerobic. They tested positive for oxidase and catalase, were susceptible to colistin, and grew at 36°C, 25°C, and 42°C.

Automated routine identification showed twice that this bacterium was in the *Moraxella* group (probability 95%). API 32GN identified the bacteria at 24 h as *Methylobacterium mesophilicum* (doubtful identification: 98.4%, with low typicity index [T] = 0.39) and at 48 h as *Acinetobacter, Pseudomonas,* or *Achromobacter* (nonreliable identification). API 20NE identified the bacteria (profile code 1001067) with weak discrimination as *Achromobacter denitrificans* (31.1%; T = 0.59) or *B. bronchiseptica* (67.1%; T = 0.66). RapID NH gave *Haemophilus influenzae* as first choice (inadequate identification; probability 76%; bioscore 1/610).

At 48 h to 72 h, cultures on BGA medium showed small, nonhemolytic colonies ≈1 mm in diameter, which were not producing any brown pigment. These tested negative for urease production. The spectra obtained gave a good identification of *B. petrii* (identification agreement 55.4%).

The sequencing of the 16S rRNA confirmed the MALDI-TOF identification. The 16S sequences showed 99% of similarity with the 16S rRNA gene sequences from the type strain of environmental origin and the other isolate from human origin. We then performed the sequencing of *risA* and *ompA* genes to compare with the genes of the other *B. petrii* isolates. The *risA* gene sequences of the clinical isolates showed 93% similarity with the type strain of environmental origin. The species with the next highest similarities were *B. bronchiseptica*, *B. parapapertussis,* and *B. pertussis,* all with a nucleotide identity of 87%. The *ompA* gene sequences of the clinical isolates demonstrated 89% similarity with the type strain of environmental origin. The species with the next highest similarities were *B. bronchiseptica*, *B. parapapertussis*, and *B. pertussis,* all with a nucleotide identity of 86%. The

weaker score obtained with *ompA* was due to an insertion of 12 nt versus the environmental strain, nucleotides not present in the other isolates from human origin.

The data regarding antimicrobial drug susceptibility obtained are shown in Table 2. Results are compared with other data available from the literature.

As shown in Figure 1, the PFGE patterns obtained with the DNA from the 3 isolates of the reported human case-patient are identical, whereas the patterns obtained with the DNA of the other isolate from human origin and the isolate from the environment show several differences. This indicates that the isolates of the present study are related but part of a different PFGE group.

Results were negative for Fim2 and Fim3 by using the agglutination technique as were results for FHA, PRN, PT, and AC-Hly by Western blot (data not shown) with specific antibodies. As shown in Figure 2, the serum of the patient infected with *B. petrii* recognized several proteins in the bacterial suspensions of the 3 *B. petrii* isolates collected from the patient. The same proteins are recognized in the bacterial suspensions of the 2 other *B. petrii* isolates of clinical and environmental origin, except for 1 low-molecular-weight protein. Most of these proteins, with small differences in molecular weights, are also recognized in bacterial suspensions of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica,* and *B. holmesii*, except for 1 protein. The pools of serum samples from patients infected with *B. pertussis* and *B. bronchiseptica* recognized high-molecular-weight proteins expressed by *B. pertussis, B. parapertussis,* and *B. bronchiseptica* in the bacterial suspensions of these 3 species. However, they did not recognize these high-molecular-weight proteins in the bacterial suspensions of *B. petrii* and *B. holmesii*. Finally, the pool of serum samples from patients infected with *B. bronchiseptica* recognized 1 protein in the *B. pertussis, B. parapertussis, B. bronchiseptica* and *B. holmesii* bacterial suspensions but not in the *B. petrii* bacterial suspensions.

In terms of cytoxicity, *B. pertussis* and *B. bronchiseptica* are cytotoxic for the J774-A1 macrophages. However, none of the *B. petrii* isolates were cytotoxic.

Discussion

Identification of *B. petrii* is still a major problem for clinical laboratories that use automated or manual identification systems. As suggested by Zbinden et al. (*37*) isolates that do not give a 99% or better typing result should be typed by 16S rRNA sequencing or MALDI-TOF. The spectra obtained here with MALDI-TOF gave

Table 2. Antimicrobial drug susceptibility testing for *Bordetella petrii* isolates, by strain and strain type, October 2007–November 2008*

Drug	FR 3799, clinical†	FR3891, clinical†	FR 3996, clinical†	FR 3497, clinical (*17*)	KMRW, environmental (*1*)	Fry et al., clinical (*16*)‡	Stark et al., clinical (*18*)
Penicillin	>32	>32	>32	>32	>32	>32	ND
Amoxicillin	>256	>256	>256	>256	8	>256	+clav: 4
Piperacillin	16	12	12	256	0.25	ND	ND
Piperacillin and tazobactam	16	48	24	256	0.38	2	≤4
Cefotaxime	>32	>32	>32	>32	>32	>32	Ceftriax: ≥64
Ceftazidime	48	256	>256	2	4	32	16
Ertapenem	>32	>32	>32	0.016	0.047	>32	ND
Imipenem	3	4	8	1	0.75	>32	ND
Meropenem	12	>32	12	0.023	0.047	>32	≤0.25
Doripenem	32	>32	>32	0.094	0.125	ND	ND
Gentamicin	1	1	1.5	12	3	4	4
Tobramycin	1	1	1	64	4	16	2
Amikacin	8	16	16	64	48	>256	8
Levofloxacin	16	24	24	2	0.25	ND	ND
Ciprofloxacin	>32	>32	>32	4	0.38	>32	2
Moxifloxacin	12	12	12	1.5	0.032	ND	ND
Minocyclin	0.75	0.75	0.75	0.5	0.125	ND	ND
Tygecyclin	0.75	1	1	0.25	0.023	ND	ND
Cotrimoxazole	0.50	0.50	0.50	0.012	0.006	8	ND
Fosfomycin	4	8	8	6	12	ND	ND
Quinupristin and dalfopristin	>32	>32	>32	>32	>32	ND	ND
Rifampin	>32	>32	>32	>32	>32	>32	ND
Daptomycin	>256	>256	>256	>256	>256	ND	ND
Linezolid	>256	>256	>256	>256	>256	ND	ND
Clindamycin	>256	>256	>256	>256	>256	>256	ND
Fucidic acid	>256	>256	>256	>256	>256	ND	ND

*Values are MICs by Etest in μg/mL. +clav, in combination with clavulanic acid; Ceftriax, ceftriaxone tested instead of cefotaxime; ND, not determined.
†This study.
‡Ampicillin was tested instead of amoxicillin.

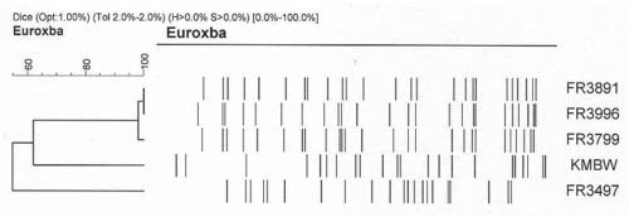

Figure 1. Genomic analysis of *Bordetella petrii* isolates chromosomal DNA profiles obtained after digestion with *Xba*I. Identity of the isolates is indicated.

an acceptable identification of *B. petrii* (identification agreement 55.4%). This is a good score, especially because the database contains only 5 spectra of this recently described species because of the low number of isolates available. The phenotypic characteristics of the isolates in our study are similar to those of the few isolates that have been previously described (*1*,*16*–*19*).

In a previous study on *B. bronchiseptica*, we and others working on *Bordetella* spp. (*16*) determined that the results obtained for many antimicrobial drugs using the disk diffusion method correlated poorly with clinical therapeutic results and with MICs established using the reference method (*32,33;* A. Le Coustumier, unpub. data). Fry et al. (*16*) reported that the clinical isolate was apparently susceptible, by disk diffusion tests, to 5 antimicrobial drugs: clarithromycin, erythromycin, gentamicin, ceftriaxone, and piperacillin+tazobactam. However, the respective reference MICs indicated that only piperacillin+tazobactam was active in vitro with a MIC of 2 μg/mL. Based on the preliminary results the patient received a 6-week course of oral clarithomycin treatment. Despite the successful clinical outcome, the isolate was subsequently shown to be resistant to clarithromycin in vitro. In the only other report (to our knowledge) on a clinical *B. petrii* isolate, MICs were determined by using VITEK2 Compact (bioMérieux) but MICs of drugs for *Bordetella* spp. cannot be determined from this database (*18*). Using Etest strips, a method that has been validated on a wide range of glucose fermenting and nonfermenting gram-negative bacteria, we determined the MIC for 26 widely used antimicrobial drugs from the main therapeutic families (*38*).

All of the 5 isolates in the present study as well as the isolates described by Fry et al. (*16*) and Stark et al. (*18*) appear to have resistance to penicillins (penicillin, amoxicillin), cephalosporins (especially third-generation, extended-spectrum cefotaxime or ceftriaxone and ceftazidime), clindamycin, quinupristin and dalfopristin, rifampin, linezolid, daptomycin, and fucidic acid. We also observed that aminoglycosides had only moderate activity against the bacteria. The isolates in our study also displayed in vitro sensitivity, but low level MICs, to minocyclin, tygecyclin, cotrimoxazole, and fosfomycin.

The large gap in the MICs of amoxicillin and piperacillin between the only environmental isolate available for this study and the clinical isolates may reflect the inducible response to exposure of the clinical isolates to formerly widely used treatment with β-lactams. Tazobactam does not restore the activity of piperacillin, or even degrade it, probably because of the induction of β-lactamase.

In contrast with the isolate of Fry et al. (*16*) and the environmental isolate (*1*), the MICS of carbapenems and systemic fluoroquinolones were high for the isolates from our patient. No previous treatment with carbapenems could be documented from the long medical history of our patient, although fluoroquinolones had been frequently prescribed for bronchiectasis. This lack could be partly due to an impermeability-linked cross-resistance between these 2 chemically unrelated families with the common porin mutation, as frequently has been observed for *Pseudomonas aeruginosa*.

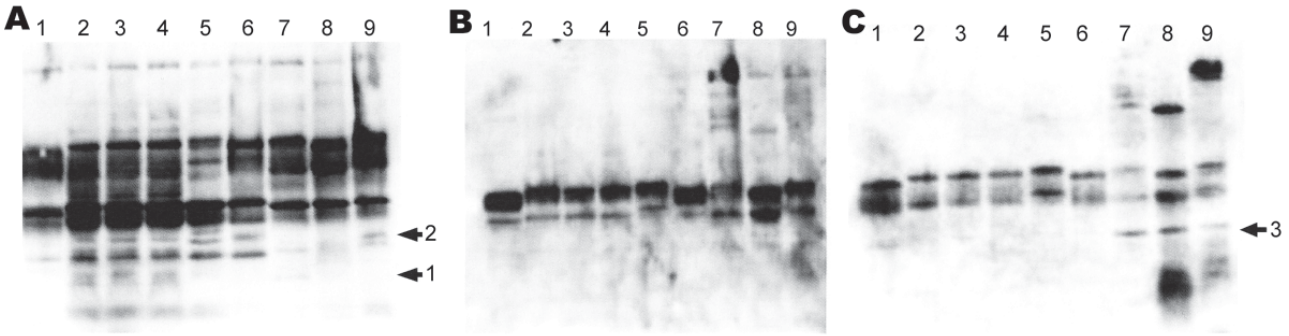

Figure 2. Western blot analysis of 10 μL of bacterial suspension (1.8×10^{10} CFU/mL) loaded to a gel and subjected to electrophoresis. The proteins were transferred onto a nitrocellulose membrane, which was incubated in mouse or human serum as described in Materials and Methods. Serum samples used were convalescent-phase serum of the *Bordetella petrii*–infected patient (A), a pool of serum specimens from *B. pertussis*–infected patients (B), and a pool of serum specimens from *B. bronchiseptica*–infected patients (C). Lane 1, *B. holmesii*; lane 2, *B. petrii* FR3799; lane 3, *B. petrii* FR3891; lane 4, *B. petrii* FR3996; lane 5, *B. petrii* FR3497; lane 6, *B. petrii* KMBW; lane 7, *B. pertussis* 8132; lane 8, *B. parapertussis* 12822; lane 9, *B. bronchiseptica* RB50. Arrows indicate the proteins specifically recognized by the anti-serum.

Using PFGE, we observed that the patterns of the DNA restriction fragments for the different isolates collected from the reported human patient were quite similar. This finding confirms the persistence of the same isolate inside the host. However, several differences are observed with the patterns of the DNA from the environmental or human isolates. These differences could be linked to the loss of pathogenic islands in some of the isolates, as has been recently reported (*22*).

Using murine serum samples specific to the major virulence factors expressed by *B. pertussis* and pool of sera from patients infected with either *B. pertussis*, *B. bronchiseptica*, *B. holmesii*, or the serum of the current patient infected with *B. petrii*, we confirmed that *B. petrii* isolates do not express FHA, Fim2 and Fim3, PRN, PT, and AC-Hly. The serum sample from the patient infected with *B. petrii* recognized only 1 protein specific to the *B. petrii* bacterial suspensions derived from the isolates of the clinical patient described in this study. Another protein was specific to the 5 *B. petrii* isolates.

None of the *B. petrii* isolates were cytotoxic for macrophages. This result was likely because these isolates do not express AC-Hly or BteA.

The source of infection and the pathogenic role of *B. petrii* are still unknown. For the study patient, the source of infection, just prior to the first episode, was most likely a contamination that occurred during the aerosol therapy performed at home under poor hygienic conditions (according to the patient). This was potentiated by local corticotherapy.

The prevalence of *Bordetella* spp. within the cystic fibrosis population may well be underestimated, due to the slow growth of this microorganism. However, the prevalence may also be underestimated for all immunosuppressed patients, particularly the elderly. The role that *Bordetellae* spp. such as *bronchiseptica* and *petrii* may play in the progression of pulmonary disease remains unknown, and these species can be misidentified in hospital laboratories (*19*).

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Dr Le Coustumier is a clinical microbiologist at Centre Hospitalier, Cahors, France. His research interests include human *B. bronchiseptica* infections and the study of nosocomial infections, particularly those caused by methicillin-resistant *Staphylococcus aureus*.

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Effects of Hand Hygiene Campaigns on Incidence of Laboratory-confirmed Influenza and Absenteeism in Schoolchildren, Cairo, Egypt

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To evaluate the effectiveness of an intensive hand hygiene campaign on reducing absenteeism caused by influenza-like illness (ILI), diarrhea, conjunctivitis, and laboratory-confirmed influenza, we conducted a randomized control trial in 60 elementary schools in Cairo, Egypt. Children in the intervention schools were required to wash hands twice each day, and health messages were provided through entertainment activities. Data were collected on student absenteeism and reasons for illness. School nurses collected nasal swabs from students with ILI, which were tested by using a qualitative diagnostic test for influenza A and B. Compared with results for the control group, in the intervention group, overall absences caused by ILI, diarrhea, conjunctivitis, and laboratory-confirmed influenza were reduced by 40%, 30%, 67%, and 50%, respectively ($p<0.0001$ for each illness). An intensive hand hygiene campaign was effective in reducing absenteeism caused by these illnesses.

Acute respiratory infections (ARIs) and diarrheal diseases cause substantial illness and death worldwide. Most of the estimated 5.5 million deaths associated with ARI and diarrhea occur in children from resource-limited countries. In these settings, where access to health services is often lacking, effective prevention methods are paramount. ARIs cause >4 million deaths annually and account for >7% of global deaths (*1*). Many ARIs are caused by viruses, including influenza A and B. Influenza viruses circulate in Egypt all year, peaking in winter months (*2*). Although influenza is generally self-limiting, each year it causes 3–5 million cases of severe illness and up to 500,000 deaths worldwide (*3*). The greatest number of excess deaths occur in persons >65 years of age, yet influenza greatly affects schoolage children as well. In addition, schoolchildren play a key role in transmission of influenza during community epidemics (*4*). Slowing or preventing transmission of influenza viruses among children may diminish the explosive transmission pattern that often characterizes annual influenza epidemics (*5*).

Diarrheal disease is the second leading cause of childhood illness and death and is responsible for ≈2 million deaths annually in children <5 years of age (*6*,*7*). Frequent and prolonged episodes of nonfatal diarrhea can lead to malnutrition, stunting of growth, and absenteeism in schoolage children (*8*,*9*). In Egypt, where child mortality rates have been reduced in recent years, diarrheal diseases still account for 13.9% of deaths in children <5 years of age. Much of the reduction in deaths caused by diarrheal diseases has resulted from better case management, including use of oral rehydration therapy and improved water and sanitation. Although deaths have decreased, the incidence of diarrheal diseases has remained relatively unchanged (*10*).

Hand hygiene is a key intervention for reducing transmission of ARI and diarrhea in community settings.

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Hand hygiene, using antibacterial soap or alcohol-based sanitizers, has been reported to result in notable reductions in the incidence of diarrheal diseases (*11*). Hand hygiene has also been specifically recommended for prevention of diseases with pandemic potential, such as severe acute respiratory syndrome and for influenza A pandemic (H1N1) 2009 (*12–14*). The objectives of this study were to measure the effectiveness of an intensive hand hygiene intervention campaign in reducing the incidence of absenteeism caused by illness and the incidence of laboratory-confirmed influenza in schoolchildren in Egypt.

Methods

Design

We conducted a randomized controlled trial to assess the effectiveness of an intensive hand hygiene campaign in reducing the absenteeism of schoolchildren due to illness, student in-class reported illness, and laboratory-confirmed influenza. The primary outcome measure was a determination of the rates of absenteeism caused by influenza-like illness (ILI) and laboratory-confirmed influenza, in which absenteeism caused by diarrhea and conjunctivitis were considered secondary outcomes. The study was performed over a 12-week period, February 16–May 12, 2008.

School Settings

Cairo Governorate was chosen because of the continuous availability of water in school settings. The socioeconomic characteristics of families sending children to government schools in Cairo are homogenous in terms of education, income, and home sanitation facilities (*15*). Most schools in Cairo have 1 large restroom with ≈10 sinks and an additional 8–10 sinks on the playground. No sinks are available in the classrooms. Average size of each classroom is ≈48 m^2 with 69 students (≈0.7 m^2/student). Before the campaign, neither soap nor hand-drying material was available in the schools. Handwashing, if done at all, was only performed by rinsing hands in water. Hands were typically dried on clothing or air-dried.

Sample Size

The sample size was calculated with the intent to detect a 20% reduction in laboratory-confirmed influenza in the intervention group, using a rate of 1.5% of laboratory-confirmed influenza per week in the control group and 70% participation. After doubling the sample to adjust for clustering, a total of 27 schools per group were adequate to detect this difference in laboratory-confirmed influenza with 80% power and 95% confidence. (The formula used to estimate the sample size is available at www.openepi.com/Menu/OpenEpiMenu.htm.) Sixty elementary schools (30 intervention and 30 control schools) were randomly selected from a numbered list of all 725 government elementary schools in Cairo by using a computer-generated random number table. All children at the intervention schools, regardless of grade, were included in the hand hygiene campaign activities, but absenteeism and illness data were only collected from children in the first 3 primary grades.

School Teams

At each intervention school, a hand hygiene team composed of 3 teachers (social studies, arts, sports) and the school nurse was established. The hand hygiene team ensured that all predesigned activities for the hand hygiene campaign were implemented on a weekly basis (Table 1). The school nurses and teachers were trained to interview students, collect absenteeism data, interview parents, and complete data collection forms, and the nurses were trained to collect and process nasal swabs to test for influenza. At control schools, the nurses were supported by a single surveillance officer who was assigned to complete data collection forms.

Although the methods for providing soap varied among the intervention schools, parents usually sent children to school with a small bag containing bar soap and a clean towel. If families could not afford soap and hand-drying material, the school administration provided them.

Six independent social workers visited the schools weekly; each visited 10 schools per week (5 intervention and 5 control schools). During each visit, they observed hand hygiene activities, soap and drying material availability, and the process of students washing their hands during the school day; they also verified the accuracy of the illness data collected by teachers.

Intervention Communication Campaign

An intensive campaign to promote hand hygiene was launched in the intervention schools to raise the awareness of students, teachers, nurses, and parents regarding the importance of hand hygiene and to increase the proportion of students washing their hands. Hand hygiene teams required students in the first 3 primary grades to wash their hands at least twice during the school day for ≈45 seconds, followed by proper rinsing and drying with a clean cloth towel.

Campaign materials were developed for 3 groups (students, teachers, parents). The teachers' guidebook included a detailed description of the students' activities and methods to encourage students to practice these activities. Posters were placed near sinks in the classrooms and on the playground. The primary message was to wash hands with soap and water upon arriving at school, before and after meals, after using the bathroom, and after coughing or sneezing.

Table 1. Hand hygiene campaign activities in intervention schools, Cairo, Egypt, February 16–May 12, 2008

Exercises, by week	School-based activities	Specific school initiatives
1. Game to explain germ theory	Obligatory handwashing twice daily for students under supervision	Handwashing champion of the week
2. How to wash hands	Soap made available	Theater play
3. Puzzle on handwashing	Morning broadcast	Best article contest on hand hygiene
4. Drawing portrait of person covering nose and mouth	Hand-washing songs	Best speech contest on hand hygiene
5. Drawing with soap bars	Parents' school meeting	Best drawing contest on hand hygiene
6. Soap bubbles game	Students-parents information transfer	Best singer contest
7. When to wash hands (open discussion)	Morning aerobics using handwashing songs	Best school contest
8. How to escape from the germs (game)		Formulation of handwashing committee
9. Germs transmission (game)		School trip to a soap plant
10. Where do germs live? (experiment)		School trip to a water purification plant
11. How sneezing contaminates hands (game)		
12. Germs characters (game)		

Grade-specific student booklets were developed; each included a set of 12 games and fun activities that promoted handwashing. At least 1 activity was used each week. A special song to promote hand hygiene was developed and played regularly at schools. Informational fliers were distributed to parents to reinforce the messages delivered at the schools. Many schools were creative in motivating students to comply with washing hands, such as selecting a weekly hand hygiene champion, developing theater plays, and launching school contests for drawings and songs.

Data Collection and Illness Definitions

Data were collected for 12 weeks during February–May 2008. The regulations of the Ministry of Education require schools in Egypt to record absences each day in a school log book, classified as absence caused by either an illness or a non-illness. The hand hygiene teams visited each classroom to verify the information collected by the school administration. They also telephoned parents of children absent due to illness on the first day of absence and interviewed them to complete an absenteeism data collection form that included specific symptoms of illness. Symptoms and signs of illness and detailed case definitions were the same as those used by Bowen et al. (*16*).

A student episode of absence caused by illness was defined as a student who was absent for any number of consecutive or nonconsecutive days during 1 calendar week with symptoms affecting the same organ system. The incidence of absence due to illness was defined as the number of absences caused by illness per 100 student-weeks. Rates of absence caused by illness with specific symptoms or signs were calculated as the number of absences due to illness associated with specific symptoms per 100 student-weeks. The incidence of in-class illness was defined as the number of reported in-class episodes of illness among the first 3 primary grade students per 100 student-weeks.

Children who were absent from school because of ILI (defined as fever ≥38°C and either cough or sore throat) were approached by the school nurse, who either visited the child at home (if possible) or asked the child to visit the school clinic if they returned to school within 3 days of absence. In addition, students who became ill during the school day were referred by teachers to the school clinic nurse. The school nurse interviewed the students and completed the in-class illness data collection form that included the same symptoms and signs used on the absenteeism data collection tool.

Laboratory Methods

Nurses collected a nasal swab from children who visited the school clinic with ILI. Nasal swabs were collected by inserting and rotating a sterile swab into the anterior nares; the specimen was then tested for influenza A and B viruses by using QuickVue, a rapid, point-of-care antigen detection test designed for use by nonlaboratory

Table 2. Incidence of absences caused by illness and reasons for absence in control and intervention schools, Cairo, Egypt, February 16–May 12, 2008*

Absence caused by illness	Control, n = 282,832 student-weeks		Intervention, n = 250,584 student-weeks		Reduction, %	p value
	No. episodes	Median (IQR)	No. episodes	Median (IQR)		
Overall illness	19,094	7.2 (3.3–9.5)	13,247	5.7 (3.4–7.6)	21	<0.0001
ILI	1,671	0.5 (0.3–1.1)	917	0.3 (0.1–0.7)	40	<0.0001
Diarrhea	1,316	0.3 (0.1–0.6)	639	0.2 (0.0–0.5)	33	<0.0001
Conjunctivitis	1,214	0.3 (0.1–0.6)	530	0.1 (0.0–0.4)	67	<0.0001

*IQR, interquartile range; ILI, influenza-like illness.

personnel (QuickVue; Quidel Corp., San Diego, CA, USA). This test was conducted only for students who had prior written approval of a parent.

Ethics and Informed Consent

The study protocol was reviewed and approved by the US Naval Medical Research Unit No. 3 (NAMRU3) Institutional Review Board (Protocol #NAMRU3.NAMRU3.2007–0007). A written letter describing the purpose of the study signed by the school principal of each school was distributed to the parents or guardians of all students in the first 3 primary grades. In addition, consent for obtaining a nasal swab from students reporting ILI was sought. Control schools received the same intervention program at the end of the study.

Statistical Methods

To adjust for the cluster design effect, we calculated the rates of absenteeism and illness separately for each school. This was performed by dividing the number of episodes of absenteeism or illness by the number of student-weeks. The answer was then multiplied by 100 to obtain rates per 100 student-weeks. Since the rates were not normally distributed, the medians of the mean rates for the intervention and control schools were compared by using the Wilcoxon rank-sum test; p values <0.05 were considered significant.

Results

During the 12-week observation period, 20,882 students (282,832 student-weeks of observation) were enrolled at the intervention schools in the first through third grades; 23,569 students (250,584 student-weeks) were enrolled at the 30 control schools. All parents approved providing information about their children's illnesses; however, 7,112 parents (16%) did not give permission for the collection of nasal swab specimens from their children, and the distribution was similar in both groups ($p>0.05$). No significant differences were found for the 2 groups in median age (8 years), sex distribution (51% male), or the median number of students per school (635 [interquartile range 394–978]).

One-week baseline data were collected for intervention and control schools 2 weeks before the hand hygiene intervention activities. This step was carried out to test data collection procedures and ensure the collection of good quality data. No significant difference was found between the intervention group and control group in the rate of absenteeism caused by ILI (relative risk 1.1, 95% confidence interval [CI] 0.9–1.4) or in the rate of confirmed influenza episodes (relative risk 0.8, 95% CI 0.5–1.5).

During the 12-week observation period, 19,094 absences caused by overall illness were reported at the control schools (7.2 absences/100 student-weeks), compared with 13,247 absences in intervention schools (5.7 absences/100 student-weeks) ($p<0.01$). Across all schools, the overall reduction in absenteeism caused by illness was 21% in the intervention schools compared with the control ($p<0.05$). Absences caused by ILI, diarrhea, and conjunctivitis were reduced by 40%, 33%, and 67%, respectively, in the intervention group. No difference was observed for in-class reported illnesses between intervention and control schools; control and intervention schools reported 6,538 and 6,028 in-class illnesses, respectively (Table 2).

Incidence of absence (per 100 students) caused by ILI was lower in the intervention group than in the control group for weeks 1–4, 5–8, and 9–12. During the first 4-week period, the 2 groups showed no differences in absence incidence caused by diarrhea and conjunctivitis, although the incidence of absence caused by these conditions was significantly lower in the intervention group for weeks 5–8 and weeks 9–12 (Figure 1).

During the 12-week observation period, 4,259 students were diagnosed with ILI in the control and intervention schools (n = 2,552 and n = 1,707, respectively). In the control schools, 881 cases (34.5%) of ILI were identified through in-class reported illness and 74.2% of these students (n = 654) were tested for influenza, of which 34.9% (n = 228) were positive. In contrast, 790 cases (46.3%) of ILI in the intervention schools were identified through in-class reporting, and while a similar proportion was tested for influenza virus (77%) (n = 609), only 18.4% (n = 112) were positive for influenza virus ($p<0.01$). In control schools, 65.5% (n = 1,671) of students were absent caused by ILI, of which 11.6% (n = 194) were tested and 27.3% (n = 53) were positive for influenza virus. In the intervention schools, ILI was responsible for 53.7% (n = 917) of absenteeism. Of these 917 students, 199 (21.7%) were tested and only 6.5% (n = 13) were positive for influenza virus (Figure 2).

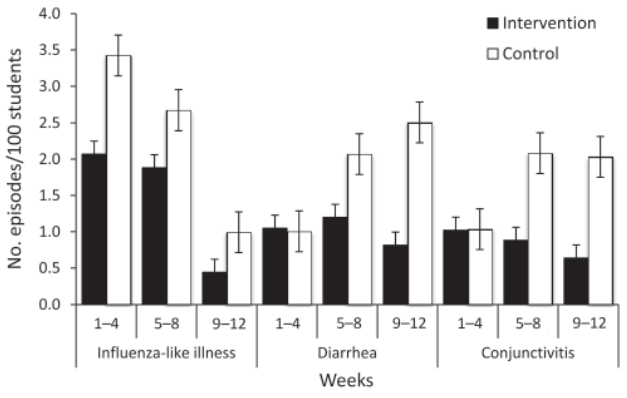

Figure 1. Episodes of absence because of influenza-like illness, diarrhea, and conjunctivitis in the intervention and control schools, by weeks, Cairo, Egypt, February–May 2008. Error bars indicate SEM.

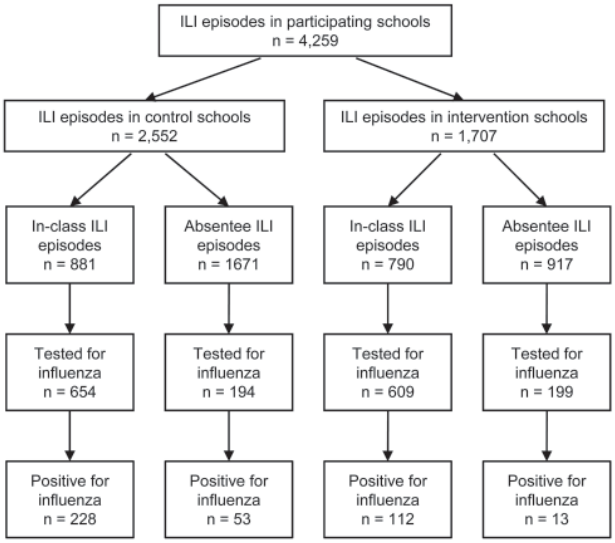

Figure 2. Diagram of results of influenza testing for students with influenza-like illness (ILI) in intervention and control schools, Cairo, Egypt, February–May 2008. Testing was done with QuickVue Rapid Antigen Test (Quidel Corp., San Diego, CA, USA).

The incidence of laboratory-confirmed influenza (A and B) per 1,000 students was significantly lower in the intervention group than in the control group over the 12-week observation period as well as during weeks 1–4 ($p<0.01$) 5–8 ($p<0.001$), and 9–12 ($p<0.001$) (Figure 3). The incidence of influenza A in the intervention group was significantly lower during weeks 1–4 and 5–8, with no statistical difference in weeks 9–12. The incidence of influenza B was significantly lower in the intervention group during weeks 5–8 and 9–12.

The largest number of confirmed cases reported from any single school was from a control school where 66 cases were reported (7.24 cases/1,000 student-weeks). Overall, control schools were 1.6× (95% CI 1.1–2.2) more likely to report at least 1 confirmed case of influenza and 2.8× (95% CI 1.7–4.7) more likely to report multiple confirmed cases of influenza than intervention schools.

The monitoring teams observed during their regular visits that ≈93% of the students in the intervention schools had soap and drying material available. All intervention schools (except 2) had a rigorous system of ensuring that schoolchildren were washing their hands at least twice daily.

Discussion

Elementary schoolchildren are important vectors for spreading infectious diseases between themselves, their families, and their communities, especially in developing countries where public schools are extremely overcrowded. Aiello et al. noted that infectious agents that children contract in schools can result in infections in up to 50% of household members (*17*).

Influenza transmission dynamics and potential methods for control are of particular interest in Egypt where avian influenza (H5N1) is endemic in poultry facilities, both commercial and backyard (*18*). Concern persists regarding the potential for recombination between seasonal influenza and subtype H5N1 strains with resultant rapid transmission of the recombinant strain, especially among high-density populations such as public school students and staff. In addition, nonpharmaceutical interventions, especially hand hygiene to mitigate pandemic (H1N1) 2009 disease spread, have been advocated by international organizations (*19*).

This randomized controlled intervention trial replicates well-known findings that intensive hand hygiene campaigns are highly successful in reducing absenteeism caused by illness and absenteeism caused by to ILI and diarrheal diseases among schoolchildren (*16*,*20*–*22*). This study also duplicates recent findings that incidence of in-class reported illness is not significantly decreased by promotion of hand hygiene (*16*). However, this study also demonstrates a decrease in laboratory-confirmed influenza as a result of an intensive hand hygiene campaign.

The 21% reduction in absenteeism caused by illness in intervention schools in this study is lower than the 42% observed among schoolchildren in the People's Republic of China (*16*) and in similar US studies (*20*,*21*). These differences might be caused by the nature of the interventions implemented: ensuring a continuous free supply of soap as in China (*16*), promoting the use of alcohol-free instant hand sanitizers (*20*), or equipping classrooms with dispensers containing alcohol as described by Guinan et al. (*21*).

In this 12-week hand hygiene trial, absenteeism caused by ILI decreased 40% and laboratory-confirmed influenza

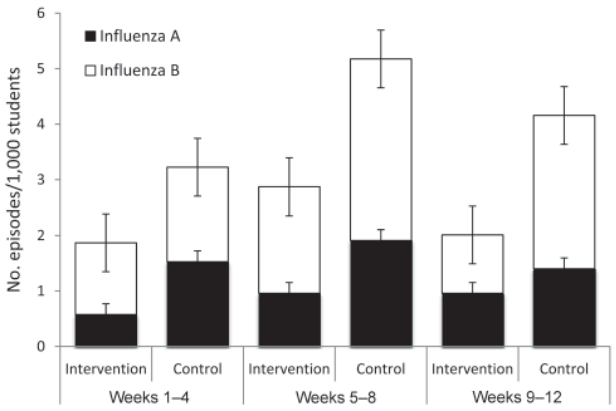

Figure 3. Episodes of laboratory-confirmed influenza A and B in the intervention and control schools, by weeks, Cairo, Egypt, February–May 2008. Error bars indicate SEM.

decreased 47% in intervention schools relative to control schools. These reductions are higher than the 21% and 16% reductions in respiratory illness reported in 2 meta-analyses of hand hygiene interventions in community settings (*22*,*23*) and the elementary school based hand hygiene program in China (*16*). A recent study evaluating the effectiveness of hand hygiene and facemasks in preventing influenza transmission in households in Hong Kong showed reduction in influenza transmission, but the differences were not significant (*24*). Differences in study design (household-based versus school-based) or in intensity of the intervention may have contributed to the positive effects in our study. Also, the greater relative reductions of influenza in our study might be attributed to specific influenza transmission dynamics for Egypt or the season when the study was conducted. In addition, Egypt's unique hand hygiene campaign required students to wash hands at least twice during the school day, which might have had a direct influence on reducing influenza.

The incidence of absenteeism caused by diarrhea was 33% lower in schoolchildren in the intervention schools. This result is similar to a Cochrane Reviews report that handwashing reduced the incidence of diarrheal episodes in children and adults by 30% (*11*). However, our study found a greater reduction in diarrhea than did a controlled trial conducted at a single elementary school (*8*), where the intervention only focused on providing alcohol-based hand sanitizers and wipes to disinfect classroom surfaces. Higher reduction rates of absenteeism caused by diarrhea (47%) were described in community settings that used soap for handwashing (*25*).

Notably, the incidence of ILIs decreased more than did diarrheal disease (40% vs 33%) in this study. Previous studies have shown a greater reduction in diarrheal disease incidence, possibly because it is easier to adopt handwashing behaviors associated with diarrhea such as preparing/eating food, defecating, etc., relative to those associated with ILI such as washing hands after sneezing or coughing. Also of interest in this study was the marked (67%) decrease in absenteeism caused by conjunctivitis in intervention schools compared to results for control schools.

There are several important limitations in this study. First, because study teams and schoolchildren and their parents were not blinded to the intervention, underreporting of illness as a cause for absenteeism in the intervention schools may have contributed to information bias. However, a rigorous system for identifying the reasons for illness based on a standardized list of symptoms as well as regular monitoring visits did not uncover any systematic errors. Differential interest of study teams may have contributed to the low rate of testing in students who were absent because of ILI in the control schools compared to the intervention schools (12% vs 22%); however, because nasal swabs were collected only from students who returned to school within 3 days of illness onset, it is unlikely that samples tested reflected the most severe manifestation of illness. Absence incidence, defined as $\geq$1 day of absence in given week, may have been overestimated if a child were ill at the end of 1 week and at the beginning of the subsequent week; however, such overestimation is unlikely to have occurred differentially between the intervention and control schools.

The relatively short duration of observation (12 weeks) may have also led to an overestimation of effect, as participants may have been more likely to adhere to new hand hygiene behavior over a shorter period. This study was not designed to measure sustainability of effect. In addition, the use of rapid tests for diagnosis of laboratory-confirmed influenza with known low sensitivity (60% in some studies) likely resulted in an underestimation of illness in each group; this would likely bias the effects towards the null. Finally, because of delays in scientific and official approvals, the campaign was not started until the end of the influenza season; a higher baseline prevalence of respiratory and diarrheal diseases during the trial period may have led to a stronger program effect on disease-specific absenteeism.

This intensive hand hygiene intervention was effective in reducing transmission of influenza among schoolchildren and was feasible and acceptable. In spite of operational difficulties in schools, the Egyptian Ministry of Health recommended hand hygiene as a means of reducing transmission of pandemic (H1N1) 2009 and other infectious diseases on a countrywide basis by using mass media campaigns (television and radio programs) and focusing on schoolchildren. Sustaining intensive national hand hygiene programs is challenging because of the high costs of training, printing materials, and logistics involved. In addition, the lack of continuous availability of soap and water is a limiting factor in remote areas. Identifying strategies that provide national, long-term, cost-effective alternatives to promote hand hygiene is critical in preventing transmission of diarrheal diseases and emerging respiratory viruses of pandemic potential.

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with all Federal regulations governing the protection of human subjects.

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Orthopoxvirus DNA in Eurasian Lynx, Sweden

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Cowpox virus, which has been used to protect humans against smallpox but may cause severe disease in immunocompromised persons, has reemerged in humans, domestic cats, and other animal species in Europe. Orthopoxvirus (OPV) DNA was detected in tissues (lung, kidney, spleen) in 24 (9%) of 263 free-ranging Eurasian lynx (*Lynx lynx*) from Sweden. Thymidine kinase gene amplicon sequences (339 bp) from 21 lynx were all identical to those from cowpox virus isolated from a person in Norway and phylogenetically closer to monkeypox virus than to vaccinia virus and isolates from 2 persons with cowpox virus in Sweden. Prevalence was higher among animals from regions with dense, rather than rural, human populations. Lynx are probably exposed to OPV through predation on small mammal reservoir species. We conclude that OPV is widely distributed in Sweden and may represent a threat to humans. Further studies are needed to verify whether this lynx OPV is cowpox virus.

Cowpox virus (family *Poxviridae*, genus *Orthopoxvirus* [OPV]) was originally considered to infect milking cows and to have zoonotic potential. Because of its relationship to variola virus and immunologic cross-reaction, cowpox virus was used to protect humans against smallpox (*1*). Later, vaccinia virus, another OPV with unknown origin, was used as a vaccine virus through the global smallpox eradication campaign, and cowpox virus infections became less common in cattle, other animals, and humans. However, during recent decades, cowpox virus infections have reemerged in domestic cats and other animals, including wild animals in captivity (*2–4*), and have increased in humans subsequent to transmission from cats, rodents, zoo animals, and circus animals (*5–9*). Cowpox virus seems to be restricted to Eurasia, but spread of poxviruses to new regions through relocation of their natural host species is possible (*10*).

Cowpox virus was first isolated from felid species—lions (*Panthera leo*), cheetahs (*Acinonyx jubatus*), black panthers (*Panthera pardus*), ocelots (*Leopardus pardalis*), jaguars (*Panthera onca*), pumas (*Felis concolor*), leopard cat (*Prionailurus bengalensis*), Pallas cat (*Otocolobus manul*), and domestic cat (*Felis catus*)—during an outbreak in the Moscow Zoo in 1973–1974 (*11*) and subsequently from 3 cheetahs in Whipsnade Park, England, in 1977 (*12*). Since 1978, cowpox virus infections have been diagnosed in domestic cats in several European countries (*13–16*); the cats were probably infected by contact with rodents, and in some cases infections were transmitted to humans (*16–18*). Serologic surveys and detection of OPV DNA by PCR have indicated that wild rodents and shrews are likely reservoir hosts of cowpox virus in western Europe (*19–23*), although infectious virus has never been isolated from these animals.

Anti-OPV antibodies have been demonstrated in carnivores such as European lynx (*Lynx lynx*), red fox (*Vulpes vulpes*), and a brown bear (*Ursus arctos*) (*24*,*25*). Anti-OPV seroprevalence was 29% among 17 lynx from the Sarek National Park, northern Sweden, and 1% among 73 lynx from southern Finland (*25*). Taking into account the human cowpox virus cases reported from Finland, Sweden, and Norway (*16*,*23*,*26*), OPV, presumably cowpox virus, is probably widespread among wildlife in Scandinavia, having small wild rodent populations as a reservoir. Because carnivores are exposed to OPV through predation

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on rodents, virus-specific DNA or antibodies in carnivores could serve as an indicator for the epizootiologic situation in rodent populations (*25*).

To our knowledge, no cowpox virus case has been reported in free-ranging wild felids. To evaluate the possibility that cowpox virus may cause disease among Eurasian lynx, we searched for evidence of OPV infection in this large, free-ranging felid in Sweden. We used PCR to detect OPV DNA in tissue samples and compared these data with pathological findings. In addition, to address the possible effects of such viruses in the ecosystems, we searched for phylogenetic relationships between the virus infecting lynx and viruses causing clinical cowpox cases in Scandinavia.

Materials and Methods

Animals

Lynx samples were collected as part of a large study assessing the health of the free-ranging lynx population in Sweden from 1989 through 1999 and providing baseline data on diseases and parasites of the Eurasian lynx. Complete necropsy was performed on all animals shipped to the National Veterinary Institute, Uppsala, Sweden, during the study period. Samples of lung, kidney, and spleen were collected during necropsy and stored at –20°C until analysis. The main causes of death were traffic accidents and sarcoptic mange (*Sarcoptes scabiei* infestation) (*27*). Infections with common feline viruses were rare (*28*), and seropositivity to *Toxoplasma gondii* was more common among lynx from central Sweden than from farther north, where the climate is more harsh and the human population less dense (*29*). A total of 263 lynx, sampled during 1995–1999, were included in the present study.

Lynx originated from all over Sweden. We differentiated 3 main geographic regions according to human population density (Figure 1). Northern Sweden, apart from the mountain chain bordering Norway, is part of the circumpolar northern coniferous forest belt, the taiga. It is sparsely populated by humans; human populations increase toward the south and gradually decline farther from the coast. The southernmost part of Sweden belongs to the broad-leaf forest region of central Europe but is mainly cultivated. Lynx typically live in forested areas and are found throughout most of Scandinavia. They may come close to human settlements. Although their main prey are roe deer, they also prey on reindeer and sheep. Furthermore, debilitated lynx affected by sarcoptic mange and orphans searching for easily accessible food are often found around human settlements. Interactions between diseased lynx and domestic cats and dogs have been documented (*30,31*).

Most (245) animals submitted were apparently healthy (225 killed by hunters, 19 died of traumatic injury, 1 died of acute circulatory failure during anesthesia), 15 had sarcoptic mange, and 3 died of starvation caused by noninfectious or unclear etiology. Lynx killed by hunters were submitted for necropsy without their fur. Body condition was evaluated by body weight, fat deposits in the abdomen and on the heart, and appearance of the bone marrow of the femur. The animals were categorized into 3 groups: normal body condition (normal to fat, n = 230), poor body condition (low amount of fat in abdomen and on heart, n = 15), and emaciated (no abdominal fat, serous atrophy of fat deposits on heart and of bone marrow, n = 18, i.e., all diseased animals).

For 228 animals, age was determined by counting cementum annuli of a canine tooth (Matson's Laboratory, Milltown, MT, USA); for the others, it was estimated on

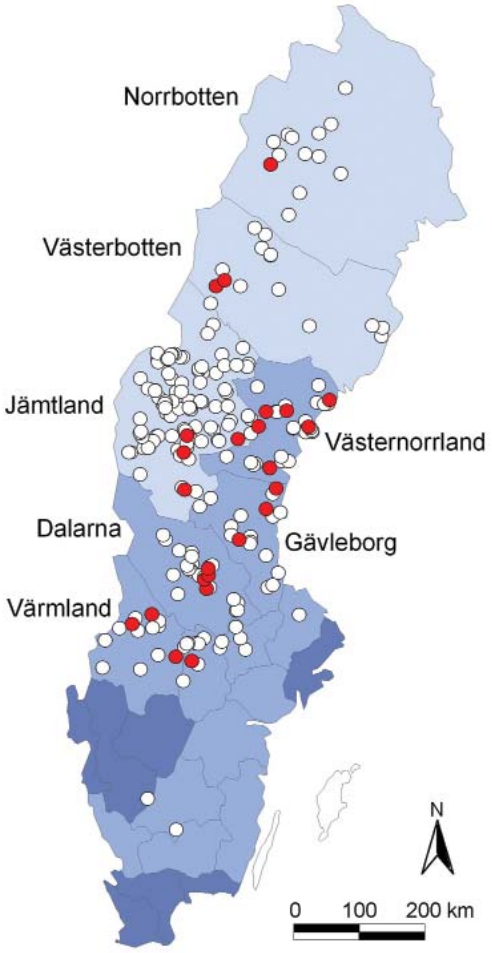

Figure 1. Geographic origin of 263 Eurasian lynx (*Lynx lynx*) collected in Sweden during 1995–1999 and tested for orthopoxvirus (OPV)–specific DNA (open circles). OPV DNA was amplified by PCR from 24 animals (9%; red circles). Light blue areas represent sparsely populated (<5 inhabitants/km^2) mountainous counties; medium blue areas represent more densely populated counties (10–41 inhabitants/km^2) farther south; and dark blue areas represent counties with the highest human population densities (>50 inhabitants/km^2).

the basis of body size and weight, tooth wear, development of genital organs, and, if appropriate, morphologic characteristics and ossification of the skull. Investigated lynx with exact age known were 0–13 years of age (mean 2.5 years ± 2.6 SD). On the basis of sexual maturity, and thus reflecting social behavior, 3 age classes were established: juvenile (≤1 year; n = 64), subadult (2 years for females and 2–3 years for males; n = 95), and adult (≥2 years for females and ≥3 years for males; n = 104). Numbers of males and females were equal in the juvenile and adult age classes, but within subadults there were more males (n = 68) than females (n = 27). According to the time of death, animals were grouped into seasons relevant to the biology of the lynx: delivery (May–July, n = 6); small kittens and lactation (August–October; n = 7); large kittens hunting with the dam (November–January, n = 24); and breeding season, separation of kittens from dam, and main hunting season (February–April, n = 224).

PCR, Electrophoresis, and Sequencing

From each sampled lynx, 25 mg lung (n = 262), 25 mg kidney (n = 263), and 10 mg spleen (n = 261) were cut in small pieces and homogenized in phosphate-buffered saline. DNA was extracted by using a QIAamp tissue kit (QIAGEN GmbH, Düsseldorf, Germany); mean DNA concentration was 39.7 μg/mL ± 23.8 SD for lung, 99.0 μg/mL ± 46.4 SD for kidney, and 101.0 μg/mL ± 49.0 SD for spleen. Five μL of the DNA eluate was used as template for the PCR.

PCR primers (MedProbe AS, Oslo, Norway) from the thymidine kinase gene (*tk*) were used as described (*21*), generating an expected PCR amplicon of 339 bp. DNA from vaccinia virus (Western Reserve; VR-119), cowpox virus (Brighton; VR-302) (both from American Type Culture Collection, Rockville, MD, USA), and cowpox virus isolated from a felid with clinical disease (*16*) was used as positive control. PCR was performed with a Gene Amp PCR System 9700 (PerkinElmer Corp., Norwalk, CT, USA). Reaction volume was 50 μL and contained 5 μL of the DNA eluate from the lynx tissue, 1 μL of each primer (25 μM), 4 μL dNTP (10 mmol/L), 5 μL $MgCl_2$ (25 mmol/L), 5 μL GeneAmp 10X Gold Buffer (Applied Biosystems, Oslo, Norway), 2.5 U AmpliTaq Gold polymerase (Applied Biosystems), and 19 μL water. The tubes were placed in a preheated block (95°C) and held for 5 min before cycling. Five cycles of denaturation (95°C for 30 s), annealing (53°C for 2 min), and primer extension (72°C for 30 s) were followed by 35 cycles with 30 s annealing time. After the last cycle, the temperature was held at 72°C for 10 min.

PCR amplicons were analyzed by using the Gibco BRL Horizon 11-14 Gel Electrophoresis System (Life Technologies, Paisly, Scotland) in a 2% agarose gel (UltraPure agarose gel; Life Technologies) with TAE buffer (0.04 M Tris-acetate, 1.0 mmol/L EDTA) and ethidium bromide for DNA staining. Fifteen μL of PCR product mixed with 3 μL 6X loading buffer (0.25% [wt/vol] bromphenol blue and 40% [wt/vol] sucrose) were loaded in each well. The gels were run in TAE buffer at 150 V for 1.5 h and examined by using a Gel Doc 2000 Documentation System (Bio-Rad Laboratories, Oslo, Norway). Primers and dNTP were removed from amplicons by using ExoSapIT reagent (Amersham Pharmacia, Uppsala, Sweden), adding 1 μL/5μL PCR product, incubating 45 min at 37ºC, and by conducting enzyme inactivation for 20 min at 80ºC. Cycle sequencing was conducted in both directions by using Big Dye 3.1 reagents (ABI BigDye Terminator version 3.1, Applied Biosystems). Two microliters EDTA (125 mmol/L), 2 μL sodium acetate (3 mol/L), and 50 μL of ethanol were added to the 20 μL sequencing product. Electrophoresis of the cycle sequencing extension products was conducted in an ABI Prism 377 DNA Analyzer (Applied Biosystems). Raw sequence data were edited by Chromas Pro software version 1.41 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) and BioEdit Sequence Alignment Editor version 7.0.4 (www.mbio.ncsu.edu/BioEdit/bioedit.html). Multiple sequence alignment was performed by using ClustalX 1.181 (*32*). The phylogenetic tree was generated with the neighbor-joining method by using MEGA 3.1 (www.megasoftware.net). The reliability of the phylogenetic relationship in the tree topology was statistically evaluated from 1,000 bootstrap replicates. The lynx orthopoxvirus DNA sequences obtained in this study have been submitted to the GenBank nucelotide sequence database (Table).

Statistical Analyses

Statistical calculations regarding differences in prevalence were performed by using NCSS 2007 Statistical Software (www.ncss.com). Statistical significance of differences was analyzed by using the 2-tailed Fisher exact test; level of significance was set at $p<0.05$.

Results

In contrast to what has been reported for domestic cats and exotic felids in zoos (*11*,*12*,*33*), no macroscopic lesions in the skin or lungs could be ascribed to a cowpox virus infection. OPV PCR amplification products of expected size (≈339 bp) were detected in tissues from 24 lynx (9.1%, 95% confidence interval [CI] 5.9%–13.3%). Mean age of the 24 lynx with positive results by PCR and exact age data was 2.5 years ± 2.6 SD. Of the PCR-positive lynx, 18 were in normal body condition, 1 showed no visible signs of disease except for otodectic otoacariasis and being in poor condition, and 5 were emaciated and had sarcoptic mange (Table). Prevalence differed significantly between

Table. Overview of 24 of 263 Eurasian lynx (*Lynx lynx)* tested for orthopoxvirus-specific DNA, Sweden*

Animal no.	GenBank accession no.	Year	Age, y†	Age class	Sex	Positive by PCR‡		
						Lung	Kidney	Spleen
17§	Not sequenced	1995	ND	Subadult	F	x	x	x
23	FJ410798	1996	1	Subadult	M		x	
31§	Not sequenced	1996	1	Subadult	M	x		
38	FJ410799	1997	2	Subadult	M		x	x
49§	FJ429238	1997	ND	Adult	F			x
104	FJ429239	1997	ND	Adult	M			x
111§	FJ429240	1997	ND	Juvenile	F		x	
124	FJ429241	1998	1	Subadult	F			x
128	FJ429242	1998	1	Subadult	F			x
163§	FJ429243	1998	0	Juvenile	M	x		
165	FJ429244	1998	2	Adult	F	x		
170	FJ429245	1998	1	Subadult	F	x	x	x
197	FJ429246	1998	5	Adult	M	x		
199	FJ429247	1998	ND	Adult	M	x		
209	FJ429248	1999	ND	Subadult	M		x	x
213¶	Not sequenced	1999	1	Subadult	F			x
214	FJ429249	1999	1	Subadult	M	x	x	x
218	FJ429250	1999	1	Subadult	M	x		x
223	FJ429251	1999	4	Adult	F	x	x	
226	FJ429252	1999	0	Juvenile	F		x	
229	FJ429253	1999	4	Adult	M		x	
255	FJ429254	1999	1	Subadult	M	x	x	
270	FJ429255	1999	6	Adult	M		x	
271	FJ429256	1999	4	Adult	M		x	

*Evidenced by PCR targeting a part of the thymidine kinase gene (*tk).* Amplicons that were sequenced are underlined. ND, not determined.
†Age determined by sectioning of teeth.
‡Lung, 11 (4.2%), kidney, 13 (4.9%), spleen, 11 (4.2%) of 263 samples positive.
§Animals with sarcoptic mange (*Sarcoptes scabiei*); all were emaciated (other orthopoxvirus-positive animals except no. 213 were in normal condition).
¶Animal in poor condition.

apparently healthy (7.7%, 95% CI 4.7%–11.7%) and diseased (29.4%, 95% CI 10.3%–55.9%; p = 0.012) lynx and between lynx in normal body condition (7.8%, 95% CI 4.7–12.1) and those that were emaciated (33.3%, 95% CI 11.8%–61.6%; p = 0.007). Most diseased and emaciated lynx had sarcoptic mange, and difference in prevalence was most significant between those with (33.3%, 95% CI 11.8%–61.6%) and without (7.7%, 95% CI 4.7%–11.7%; p = 0.007) sarcoptic mange. A significant difference was also found between areas with sparse human inhabitants (5.3%, 95% CI 1.5–12.9) and areas with higher human density (14.3%, 95% CI 8.4–22.2; p = 0.016). Yearly prevalence of animals having OPV DNA varied from 5.3% in 1997 (95% CI 1.5–12.9) to 20% in 1995 (95% CI 0.5–71.6), but differences were not significant. No significant differences were observed between sexes, age classes, or seasons.

Sequence data were obtained from 21 of 24 samples positive by PCR (Figure 2). The partial *tk* gene sequences were identical for all 21 samples. The obtained OPV *tk* gene sequences were also identical to that of a cowpox virus isolate from a person in Norway (No.H1) and differed by1 nt substitution from a cowpox virus isolate from a felid in Norway (No.F1) (Figure 2). In addition, the generated OPV *tk* gene sequences differed from those of CPXV-BR and of cowpox virus isolates from Sweden (Swe.H1 and Swe.H2) by multiple nucleotide substitutions (Figure 2). Phylogenetic analysis of the OPV *tk* gene sequences revealed that they clustered with the *tk* gene of cowpox virus No.H1 and cowpox virus GRI (*30*) but separated from that of cowpox virus isolates from Sweden (Swe.H1and Swe.H2) and from CPXV-BR (Figure 3).

Discussion

On the basis of previous serologic investigations that found anti-OPV antibodies in lynx in Sweden (*25*) and our detection of OPV-specific DNA in lynx tissue samples, we conclude that the Eurasian lynx in Sweden are infected with OPV. Thus, we report finding OPV in free-ranging, wild felids.

We used the *tk* gene as a target for the PCR because it is highly conserved within species of the genus *Orthopoxvirus* (*34*), thus enabling DNA amplification of different OPVs in the sample. We cannot rule out the possibility that the OPV *tk* sequences obtained from lynx were derived from other OPVs apart from cowpox virus; but although the phylogeny is based on a partial sequence of a single gene, which may have restrictions as a predictor of phylogenetic relationships (*35*), we suggest that the *tk* OPV sequences obtained from the lynx in this study were probably derived from cowpox virus. This assumption is

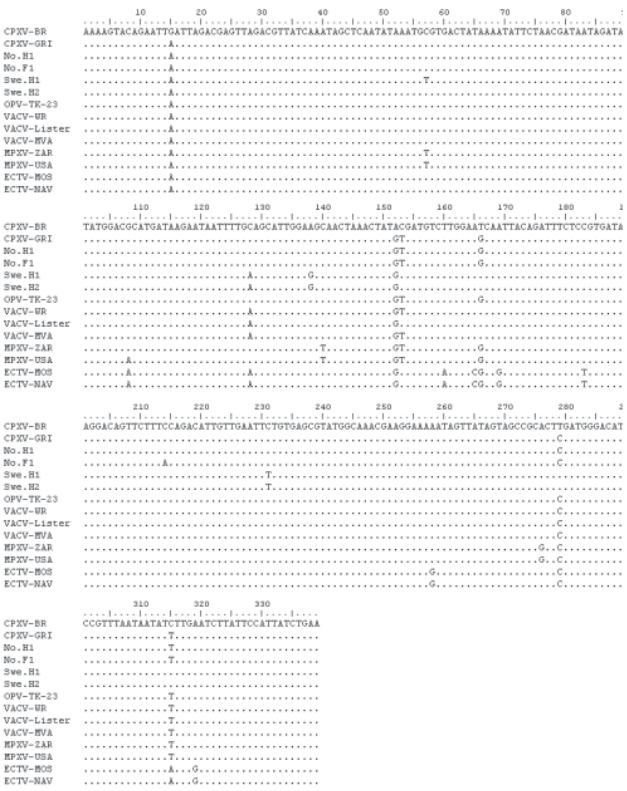

Figure 2. Multiple sequence alignment of the partial thymidine kinase (*tk*) gene obtained from Eurasian lynx (*Lynx lynx*) compared with the *tk* gene from other orthopoxviruses (OPVs). OPV-TK-23 represents all 21 sequences obtained from lynx tissues because they had 100% sequence homology. Swe.H1 and Swe.H2 represent 2 cowpox virus isolates from persons in Sweden. No.H1 and No.F1 represent cowpox virus isolates from a human and a felid, in Norway, respectively.

based on the fact that the lynx *tk* sequences were 100% identical with the partial *tk* gene of several cowpox virus isolates (Figure 2 and data not shown) and is also supported by the phylogenetic analysis, which showed that the lynx *tk* gene sequences formed a common clade with those of CPXV-GRI (strain GRI-90 isolated from a person infected by contact with a mole) (*36*) and of isolates from 1 person (No.H1) and 1 felid (No.F1) from Norway (Figure 3). Given the general genetic heterogeneity of cowpox virus isolates (*35,37*), it is not surprising that the clade containing the OPV *tk* sequences from lynx diverged from that of some other cowpox virus isolates, such as CPXV-BR and the 2 isolates from persons in Sweden (Swe.HI and Swe. H2; Figure 3).

Unexpectedly, the OPV sequences obtained from the lynx in Sweden were similar to the 2 isolates from a person and a felid in Norway and less similar to 2 isolates from persons in Sweden. A potential explanation is that none of these 4 clinical cases occurred near the sites in which the lynx were sampled for this study. In Sweden, the 2 human cases appeared in Skåne, the southernmost part of the country, 150–200 km from the southernmost region where the lynx were sampled. In Norway, all feline cases and all but 1 human case occurred in the southwestern part of the country, far from the common mountainous Sweden–Norway border, the region where most lynx are found. Recently, only 1 case of cowpox virus in a human has been identified in this region (Nordland County, Norway).

The presence of OPV DNA in ≥1 internal organs of the lynx is evidence of a virus infection and indicates that the host most likely has entered a viremic phase. No specific pathologic lesions in skin or lungs were recorded, but only a limited number of skins were submitted with the carcasses. This finding calls for increased efforts to document possible clinical signs, pathologic changes, and the effects of cowpox virus infection in lynx, taking into account the clinical signs and organ lesions documented in domestic cats and in large felid species in zoos (*11*,*12*).

Wild rodents such as bank voles, wood mice, and field voles have been suggested as the main cowpox virus reservoirs in Finland (*23*), Great Britain (*19*,*22*), and Norway (in addition to lemmings and common shrews) (*20*,*21*). Cowpox virus is considered to be endemic in these species in certain regions, circulating in several host species at the same time. In a previous examination of stomach contents of Norwegian lynx, which are part of the common lynx population inhabiting Norway and Sweden, remnants of small rodents were found in 8% of the individuals (*38*), demonstrating that rodents are part of their prey.

Domestic cats may also act as source of infection for lynx, as a potential prey species. Lynx from areas with high human density, and thus a presumably larger domestic cat population, were more often infected than lynx from less populated areas, indicating a possible relationship between domestic cats and lynx. A similar association with human presence has already been observed with regard to seropositivity to *T. gondii* in some lynx in this study (*29*). In contrast, previous studies (1999) of other pathogens in some of the lynx (n = 70) included in this study suggested that contacts between lynx and domestic cats are uncommon; seroprevalence of antibodies against feline parvovirus was low; and feline leukemia virus antigen and antibodies against feline coronavirus, calicivirus, herpesvirus, and feline immunodeficiency virus were absent (*28*). Assuming that lynx are susceptible to these feline pathogens, this difference may be explained by the fact that domestic cats are commonly vaccinated against these viral infections but not against *T. gondii* and OPVs. Nevertheless, these results may suggest only limited contact between domestic cats and lynx, pointing at wild rodents and possibly shrews as the direct source of the OPV infection for lynx.

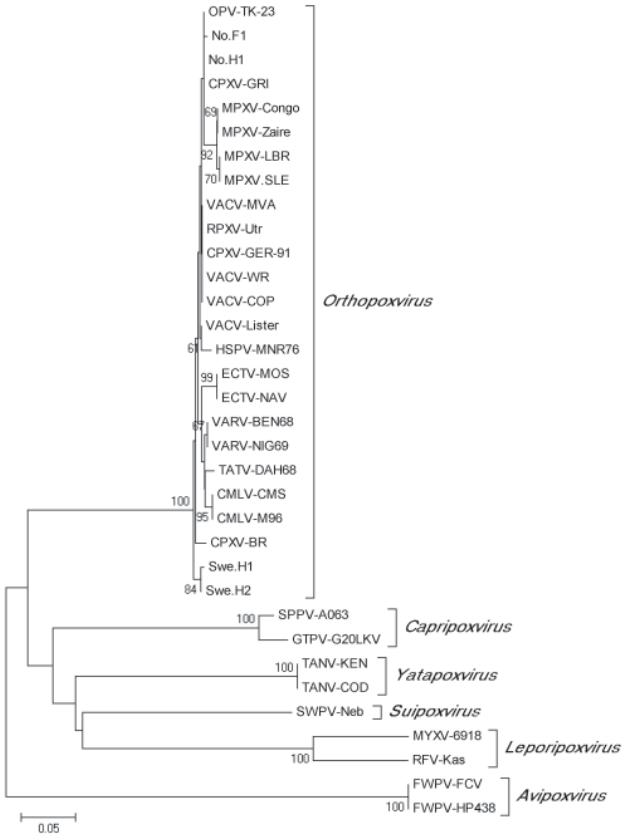

Figure 3. Phylogenetic tree (neighbor-joining method) generated from alignment of identical partial orthopoxvirus (OPV) thymidine kinase (*tk)* gene sequences obtained from 21 Eurasian lynx (*Lynx lynx*) from Sweden (designated OPV-TK-23) and corresponding sequences from cowpox virus isolates and other members of the genus *Orthopoxvirus* as well as other genera of the family *Poxviridae*. The corresponding *tk* gene sequences of 2 fowlpox viruses (genus *Avipoxvirus*) were used to root the tree. Only bootstrap values >60 are shown. Scale bar represents distances in substitutions per site.

Among the 24 animals that were OPV-positive by PCR, 5 (21%) had sarcoptic mange, whereas the total prevalence of mange among the 264 animals included in this study was 6.4%; this finding indicates a possible relationship between OPV infection and infestation with *S. scabiei.* The severe epidermal lesions and breakage of the skin barrier might predispose the animals to infection by OPVs, such as cowpox virus, and such viruses might contribute to the severity of sarcoptic mange in lynx. Alternatively, altered behavior in diseased lynx could influence exposure to infection. In contrast to healthy lynx, those with mange are commonly found in human settlements, where they sometimes prey on domestic animals, including cats and dogs (*30*).

We found no seasonal differences in OPV prevalence, although one could assume that autumn, with peak populations of rodents and shrews, would be when contact rate between these animals and lynx is highest. It could also be expected that the presence of OPVs such as cowpox virus would fluctuate between years, reflecting the fluctuations of rodent populations, but we found no significant differences in prevalence between the years in this study. These findings could be the result of the low number of samples that were included from the nonhunting seasons and from some of the years represented in the study.

Cowpox virus infection is a zoonosis, capable of being transmitted from rodents to humans, often by domestic cats. Cowpox virus infection in humans is usually characterized by single lesions on the infection site (face, hands, arms), but it sometimes spreads and cause secondary lesions and complications and can be especially severe in immunocompromised persons (*6*,*16*,*23*). To our knowledge, however, only 2 verified cowpox cases in humans have been recorded from Sweden (*16*,*26*).

In conclusion, our results support the hypothesis that carnivores that prey on OPV reservoir species can be used as indicator species for the presence of such viruses in the ecosystem (*25*). This study also provides further evidence that OPV, presumably cowpox virus, is widely distributed in ecosystems in Sweden. This finding may be relevant for vaccination strategies (*39*), especially when considering the use of OPVs as vectors in genetically recombinant vaccines and their ability to undergo spontaneous genetic recombinations with virus relatives when replicating in the same cells of a host. Moreover, our findings indicate that the role of OPVs, such as cowpox virus, as potential human pathogens may increase, considering their broad distribution in ecosystems, the cessation of smallpox vaccination of humans, and an increasing number of immunocompromised persons. Thus, targeted surveillance of rodent species, and the carnivores that prey on them, is necessary for monitoring the emergence or reemergence of these viruses as potential human pathogens. Further genetic studies are needed to determine whether the detected virus in the free-ranging lynx population in Scandinavia is indeed cowpox virus.

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Genome Sequence of SG33 Strain and Recombination between Wild-Type and Vaccine Myxoma Viruses

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Myxomatosis in Europe is the result of the release of a South America strain of myxoma virus in 1952. Several attenuated strains with origins in South America or California have since been used as vaccines in the rabbit industry. We sequenced the genome of the SG33 myxoma virus vaccine strain and compared it with those of other myxoma virus strains. We show that SG33 genome carries a large deletion in its right end. Furthermore, our data strongly suggest that the virus isolate from which SG33 is derived results from an in vivo recombination between a wild-type South America (Lausanne) strain and a California MSD-derived strain. These findings raise questions about the use of insufficiently attenuated virus in vaccination.

Myxoma virus is a member of the family *Poxviridae* and the genus *Leporipoxvirus* (*1*). It causes a benign infection in American rabbits (*Sylvilagus* spp.) but is responsible for myxomatosis in the European rabbit (*Oryctolagus cuniculus*). This systemic and lethal infection is characterized by a large myxoma at the inoculation site, a leonine facies caused by edema, and numerous secondary myxomas (*2*).

Distinct myxoma virus (MYXV) strains from South America and California have been identified; virulence of California MSW strain is higher than that of South America strains in European rabbits (*3*). In contrast, the California MSD strain is reported to be less pathogenic (*3*) and has thus been used as a basis for the generation of vaccine strains on several occasions.

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MYXV was introduced in France in 1952 as a means to control wild rabbit populations (*2*), and it has since spread widely throughout Europe. The strain used had been derived from a virulent South America strain (*4*) and has been called Lausanne since 1957 (*5*). Although MYXV was introduced to control wild rabbit populations, it rapidly spread to domestic rabbits, and by 1954, 30%–40% of the rabbit industry in France had been destroyed (*2*). Shope fibroma virus (SFV) was first used as a vaccine (*6*,*7*) but was only moderately effective. Limited trials were performed (*2*) by using an MSD-derived vaccine strain developed in California by Saito et al. (*8*), but this strain was later shown to cause myxomatosis symptoms in rabbits (*9*,*10*). Further attempts to attenuate the Saito strain were made (*9*). Some of the vaccine strains used throughout Europe today, such as Borghi (*11*) and MAV (*12*), are derived from the Saito strain.

In France, another attenuated vaccine was developed by Saurat et al. (*13*) in the École Nationale Vétérinaire de Toulouse virology laboratory. MYXV SG33 strain was obtained in 1977 by serial passages on a rabbit kidney cell line and chicken embryo cells at 33°C from an isolate obtained from a wild rabbit killed in the Toulouse area in 1973 (*13*). It has since been widely used as a vaccine against myxomatosis in rabbits in France and other countries in Europe.

Preliminary analyses of the SG33 genome showed a large deletion near the right end of the genome (*14*,*15*). Cavadini et al. recently performed a partial analysis of the SG33 sequence (*16*). They amplified and sequenced 200-bp to 10,000-bp fragments from 15 genomic locations, spanning 35 MYXV genes, and demonstrated that it was highly (97%–100% identity) similar to Lausanne. However, they

reported somewhat lower similarities between both strains for M138L-M139R (GenBank accession no. HM104692) and M142R-M144R (GenBank accession no. HM104702) sequences, with 84% and 89% identity, respectively. They observed 100% identity between their M138L-M139R sequence and the only available MSD sequence, a partial sequence of M138L (GenBank accession no. AF030894) (*17*). We present the analysis of the genome sequence of MYXV SG33 vaccine strain, which confirms the presence of a large right-end deletion and shows evidence of a field recombination between a wild-type and a vaccine strain.

Materials and Methods

Cells and Viruses

Rabbit kidney cells (RK13, ATCC CCL-37) were grown in Dulbecco modified Eagle medium (GIBCO-BRL-Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Eurobio, Les Ulis, France). Culture medium was supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin. MYXV SG33 strain was propagated in RK13 cells grown in OptiMEM (GIBCO-BRL-Invitrogen) supplemented with 2% fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Genomic DNA Preparation

MYXV-infected RK13 cells were harvested and centrifuged. The cell pellet was homogenized in TL20 (20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA; pH 8.6), disrupted in a Dounce tissue grinder, and centrifuged at 1,200 × *g* at 4°C for 10 minutes. The supernatant fluid was laid over an equal volume of a 36% sucrose cushion in TL20 and centrifuged at 200,000 × *g* for 2 hours in an SW 41 rotor at 4°C. The pellet was homogenized in TL20, laid over a 36% sucrose cushion, and recentrifuged. The new pellet was homogenized in TL20 and run into a linear 30%–65% sucrose gradient by centrifugation at 200,000 × *g* for 3 hours. The viral band was harvested and diluted in TL20 and then centrifuged at 130,000 × *g* for 1 hour. The pellet was homogenized in TL10 (1 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA; pH 8.6). After addition of 20% (vol/vol) of a 10% (wt/vol) sodium dodecyl sulfate solution, 20 µL of 10 µg/µL proteinase K of viral suspension, and 10% (vol/vol) of 20 mg/mL RNase A, the suspension was incubated at 50°C for 90 minutes with agitation. DNA was extracted by using a phenol/chloroform protocol and precipitated with 5 mol/L NaCl, 100% ethanol, rinsed with 70% ethanol, and resuspended in water.

DNA Sequencing and Sequence Analysis

SG33 genomic DNA sequencing and assembly was performed at Beckman Coulter Genomics (Danvers, MA, USA) by using the Roche (Basel, Switzerland) 454 Life Sciences GS FLX Titanium pyrosequencing platform. The borders of the terminal inverted sequences (TIR) sequences were amplified and sequenced by using an internal primer (5′-ACGTCTACGTCCGACTGTCC-3′ for the left TIR, and 5′-AGTCGCGTGGAGAAATCAAT-3′ for the right TIR) and an external primer (5′-AATT TATAGCTCTTAAAAAAAAGTATAACC-3′) corresponding to the 30 first bp of Lausanne sequence (GenBank accession no. AF170726.2) (*18*).

Sequence genome alignments were performed by using BLAST (*19*) and DNA Strider version 1.4 (*20*). The complete SG33 sequence has been deposited in GenBank under accession no. GQ409969.

Results

MYXV SG33 strain DNA was extracted from infected RK13 cells and sequenced. The generated contig was aligned to Lausanne strain genome sequence (GenBank accession no. AF170726.2) (*18*), for comparison. PCR amplification and sequencing of the most external 900 bp of each TIR showed 100% identity between both strains in these regions.

The genome of MYXV Lausanne strain was completely sequenced (*18*). Its 161.8 kbp encode 171 open reading frames (ORFs). Twelve of these ORFs are present in duplicate because of their localization in the TIRs of the genome. The left and right end regions of the genome (including the TIRs) mostly contain genes involved in the virulence of MYXV, whereas essential genes are found in the central part of the genome (*18*).

We determined SG33 genome to be 148,244 bp long, >13.5 kbp shorter than that of Lausanne, which is consistent with our data indicating a large deletion at the right end of the genome (*14*,*15*). A deletion spans from the second half of M151R gene to the end of M-T1 (M001R). It was confirmed by PCR amplification of the region and resequencing (data not shown). The consequences of the deletion are the absence of 13 genes and the in-frame fusion of the truncated M151R and M001R ORFs (online Appendix Table, www.cdc.gov/EID/content/17/4/633-appT.htm).

M151R encodes Serp2, a serpin that specifically binds interleukin-1β–converting enzyme (*23*) and is involved in the pathogenesis of myxomatosis (*24*). The deletion would result in the putative translation of a Serp2 protein in which its 176 last aa are missing and replaced by the 80 C-terminal aa of M-T1 protein, a CC-chemokine inhibitor (*25*). It was shown that the reactive site loop of Serp2 corresponds to its last 40 aa (*26*). It is thus unlikely that Serp2 retains its enzymatic activity. As concerns M-T1, its structure and function rely on an N terminal signal sequence and 8 conserved cysteine residues spread throughout the protein (*27*). Thus, as with Serp2, the remaining M-T1 protein

is unlikely to retain any activity. Furthermore, previous experiments showed that no protein could be specifically detected by an anti-Serp2 serum in SG33-infected cells (*23*), suggesting that the fusion protein is absent or unstable. A great proportion of the genes deleted in SG33 strain remain as a single copy in the left-end TIR (M002L, M003.1L, M003.2L, M004L, M005L, M006L, M007L, M008L, M008.1L), but M152R, M153R, M154R, and M156R are missing.

M152R encodes an atypical serpin, the deletion of which triggers an attenuation of virulence in rabbit, associated with the absence of secondary myxomas (*28*). M153R codes for a factor involved in MHCI and Fas-CD95 down-regulation. Its deletion induces a reduction of clinical signs and virulence in rabbits (*29*). M154R codes for a protein presenting 50% identity with M2L, a vaccinia virus gene that was shown to inhibit induction of NF-κB activation through an ERK2 pathway in virus-infected human embryonic kidney cells (*30*). Finally, M156R-encoded protein is a structural mimic of eukaryotic translation initiation factor eIF2α (*31*). Hence, all these genes seem to be involved in the virulence of MYXV, thus accounting for their combined deletion leading to a high attenuation of the strain.

Apart from this large deletion, SG33 genome presents other differences with Lausanne genome. Mutations in intergenic sequences are not discussed here. In contrast, we established a gene-by-gene comparison of Lausanne and SG33 strains. The genes presenting amino acid discrepancies are listed in the online Appendix Table.

Some of the differences observed with the genomic sequence of the Lausanne strain (online Appendix Table, M020L and M069L) have already been reported for other strains and were attributed to errors in the Lausanne genome sequence (*21,32*). They will not be further discussed.

M011L-encoded protein is involved in the regulation of apoptosis and is directed specifically to mitochondria by a short COOH-terminal region (*33*). In the SG33 genome, a substitution in M011L sequence leads to a non-sense codon and to the generation of 2 ORFs of 33 and 115 codons, respectively. The sequence surrounding the first AUG is 5′-UCGUCGAUGG-3′, which is partially divergent from KOZAK consensus (5′-gccRccAUGG-3′) (*34*) and thus consistent with the translation of the second ORF of the mRNA. The resulting protein should still have the ability to distribute in the mitochondria, because the targeting region is at the C-terminus of M11L (*33*). However, whether 1 or both of these polypeptides are actually expressed and functional remains to be clarified.

Among the other genes with major differences with regard to Lausanne, M077L is putatively lengthened by 23 N terminal amino acids because of the mutation of a stop codon upstream from the ATG (online Appendix Table). Nevertheless, this potential additional coding sequence corresponds to the promoting region of M077L and might thus not be transcribed, let alone translated.

From a global point of view, when compared with the Lausanne genome, SG33 DNA exhibits a high degree of nucleotide similarity from M000.5L to half of M135R and at the end of the right TIR (M000.5R). In these regions, 108 genes encode proteins 100% identical to their Lausanne counterparts. Among these, 91 genes have nucleotide sequences that are 100% identical. In contrast, in the same regions, only 5 complete or partial ORFs (spanning from the second half of M076R to the first third of M080R) are <97% identical to their Lausanne counterparts (online Appendix Table; Figure).

From M135R to M001R, and not taking the deletion into account, identity dropped to 75%–91% (online Appendix Table; Figure). We then compared SG33 sequence with the available partial sequences of California MYXV strains MSD and MSW. As described (*16*), SG33 is 100% identical to the only MSD sequence in GenBank, a partial sequence of M138L (GenBank accession no. AF030894) (*17*). Labudovic et al. (*22*) partially sequenced MSW strain using cloned *Eco*RI and *Sal*I fragments (GenBank accession nos. CC783373–CC783446 and CC799152–CC799159). The major difference between MSW and Lausanne strain is a duplication in the left TIR of 5 complete (M151R, M152R, M153R, M154R, and M156R) and 1 partial (M150R) ORFs from the right end of the genome, causing the partial deletion of M009L (*22*). As with Lausanne, comparison between SG33 and MSW sequences clearly shows 2 different regions in SG33 sequence (Figure). From M002L to M134R, nucleotide identity between SG33 and MSW ranges from 70.9% to 95.2%, as is the case between Lausanne and MSW sequences (*22*). SG33 M076R and M080R, which were shown to be the more divergent from Lausanne in this region, showed identity within the same range (92.7% and 95.2% identity to MSW, respectively) and are thus not closer to MSW than to Lausanne. In contrast, SG33 and the available MSW sequences from M149R to M151R share 99.4% to 99.9% identity and present only 7 differences.

Discussion

Analysis of SG33 genome sequence confirmed a 13.5-kbp deletion at the right end of the genome. Notably, only 1,045 bp of the right RIT remain in SG33 sequence. This finding suggests that such a short residual sequence is sufficient for a correct genome replication. In addition, comparison with databanks showed that SG33 seems to be a composite virus, resulting from a recombination between South America (Lausanne) and California (MSW/MSD) strains. The strain from which SG33 is derived was obtained from a rabbit killed in the Toulouse area in 1973.

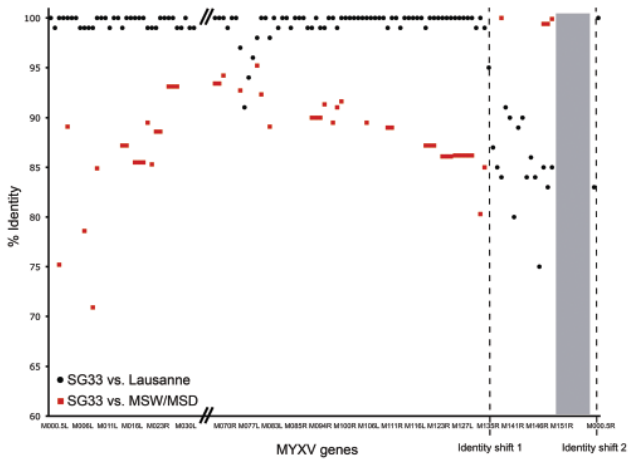

Figure. Schematic comparison of SG33 nucleic acid similarities with Lausanne and California MSD/MSW myxoma (MYXV) virus strains. Nucleotide identities were calculated between SG33 and Lausanne open reading frames and between MSW available sequences and the corresponding SG33 sequences. Dotted lines, SG33 vs. Lausanne and MSD/MSW identity shifts. Gray box, SG33 deletion.

This isolate was injected into 2 rabbits in whom classical yet delayed myxomatosis developed. One rabbit survived, and the other died 34 days after infection, which indicates that this virus was attenuated to some degree, although the number of rabbits tested is not statistically relevant. Then, serial passages on a rabbit kidney cell line and chicken embryo cells at 33°C led to the strain named SG33 (*13*).

Although somewhat attenuated, the initial viral isolate had retained enough virulence to kill rabbits. Because several genes deleted in SG33 play a critical role in virus pathogenicity, it is unlikely that this deletion was present in the initial viral isolate and is more likely the result of in vitro adaptation. It is unfortunate that this initial viral isolate was lost (R. Py and J. Gelfi, unpub. data) because sequencing of this virus would be the only way to reach a definite conclusion on this point.

However, the question of the recombination remains open. No California strain has ever been handled in the virology laboratory of École Nationale Vétérinaire de Toulouse, where SG33 was obtained (R. Py, unpub. data). In contrast, before 1970, MSD-derived Saito strain (*8*,*35*) was used for some time as a vaccine in the rabbit industry in France (*2*). It has since been demonstrated that this strain is not completely attenuated and is responsible for myxomatosis symptoms in the rabbit (*9*,*10*). It is thus possible to assume that it could disseminate and infect wild rabbits.

During the same period, Lausanne-like strains were circulating in wild rabbits in the Toulouse area. For example, the Toulouse-1 strain, which was isolated from an infected rabbit in 1952 and deposited at the Collection Nationale de Cultures de Microorganismes at Pasteur Institute (CNCM I-1592), is close to Lausanne. M151R, M152R, and M153R are 99%–100% identical to their Lausanne counterparts (*26*,*28*,*29*). Thus, the most plausible explanation of the dual origin of SG33 is that the isolate used to generate it was itself the product of a field recombination between a virulent South America strain and a vaccine California strain. The fact that the only MSD sequence available shows 100% identity with the corresponding SG33 sequence (*16*; this work), strongly supports this hypothesis.

Other occurrences of recombination of poxvirus strains have been described. It was established that malignant rabbit virus is the result of a recombination between MYXV and SFV (*36*,*37*). Nevertheless, because it was isolated from tumors induced by an uncloned stock of SFV (*38*), the recombination event most likely happened in vitro. Similarly, Gershon et al. described genetic recombination between capripoxviruses during natural transmission of wild-type strains (*39*). However, SG33 sequence might be evidence of a recombination between vaccine and virulent poxvirus strains in the field.

These findings raise the issue of the use of insufficiently attenuated live viruses, especially when used as recombinant vaccines. It was shown that loss of transgene could occur in recombinant viruses obtained from in vitro co-infection of permissive cells with a live modified vaccinia Ankara–vectored influenza vaccine and a naturally occurring cowpox virus (*40*). As previously described, the MYXV Saito strain used before 1970 was not sufficiently attenuated and was thus potentially able to disseminate and recombine with circulating wild-type strains.

Since then, SG33 and Borghi vaccine strains have been widely used in France and in Europe, and no event of virulence recovery was ever reported, which suggests that the attenuation of these strains is stable. Nevertheless, a complete sequencing of MSD strain would give clearer insight into the origin of MYXV strains now used. However, this raises the need for better knowledge of the strains used to engineer recombinant viruses, particularly at a time when poxvirus-vectored vaccines against infectious diseases and cancer are being developed.

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Shedding of Pandemic (H1N1) 2009 Virus among Health Care Personnel, Seattle, Washington, USA

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The Centers for Disease Control and Prevention (CDC) recommends that health care personnel (HCP) infected with pandemic influenza (H1N1) 2009 virus not work until 24 hours after fever subsides without the use of antipyretics. During an influenza outbreak, we examined the association between viral shedding and fever among infected HCP. Participants recorded temperatures daily and provided nasal wash specimens for 2 weeks after symptom onset. Specimens were tested by using PCR and culture. When they met CDC criteria for returning to work, 12 of 16 HCP (75%) (95% confidence interval 48%–93%) had virus detected by PCR, and 9 (56%) (95% confidence interval 30%–80%) had virus detected by culture. Fever was not associated with shedding duration (p = 0.65). HCP might shed virus even when meeting CDC exclusion guidelines. Further research is needed to clarify the association between viral shedding, symptoms, and infectiousness.

Health care personnel (HCP) with influenza infections can transmit virus to patients. This finding is of particular concern for patients with underlying medical conditions and places them at risk for serious influenza infections. Understanding the duration of shedding of pandemic (H1N1) 2009 virus detected by rapid culture and real-time reverse transcription–PCR (RT-PCR) among HCP is useful in developing infection prevention measures for the health care setting.

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The Centers for Disease Control and Prevention (CDC) created guidelines for infection control in health care settings to prevent influenza transmission from infected HCP to patients and other HCP. (*1*) These guidelines for the 2009 influenza season (2009 CDC criteria) recommend that HCP who have a fever and respiratory symptoms stay home from work for 24 hours after fever subsides without the use of fever-reducing medications. HCP who do not have a fever are permitted to work if they use appropriate infection control practices (*1*). CDC also recommends that HCP who are caring for severely immunocompromised patients (e.g., patients with hematopoietic stem cell transplantations) be considered for temporary reassignment or be excluded from work for 7 days from symptom onset or until resolution of symptoms, whichever period is longer (*1*). Earlier return to work is permitted for HCP who are caring for patients with lesser degrees of immune system compromise who also might be at increased risk for complicated influenza infections.

A limited number of studies have described the duration of pandemic (H1N1) 2009 virus shedding among healthy persons, as estimated by the presence of viral RNA detected by real-time RT-PCR or viable virus detected by culture. A study by the US Air Force demonstrated that viable virus was present in 24% of nasal wash samples from infected military trainees 7 days after symptom onset (*2*). To et al. reported that virus was undetectable by culture 5 days after symptom onset or by real-time RT-PCR at 8 days among 21 of 22 hospitalized patients treated with oseltamivir (*3*). Among household cases, Suess et al. reported mean shedding durations by real-time RT-PCR for treated and untreated patients of 5.7 days and 7.1 days, respectively (*4*).

On September 27, 2009, Public Health–Seattle and King County (PHSKC) in Seattle, Washington, was

notified of an outbreak of pandemic (H1N1) 2009 among young, otherwise healthy HCP (medical residents) who had attended a work retreat at hospital A during September 21–25. We conducted an investigation to characterize the influenza outbreak and describe viral load changes, shedding duration, and the association between these factors and fever.

Methods

On September 27, 2009, after the hospital infection prevention office at PHSKC was notified about a retreat participant with acute influenza A, all HCP who had attended the work retreat were contacted. HCP who were scheduled to be working were assessed to determine the presence of respiratory symptoms. All HCP with any respiratory symptoms were tested for influenza infection by direct fluorescent antibody (DFA) testing or real-time RT-PCR at Hospital A; negative DFA results were confirmed by real-time RT-PCR (*5*). All viruses were subtyped.

Descriptive Data

All retreat attendees were informed of the study and asked to participate. Participation in the study was voluntary. Attendees who agreed to participate completed an online questionnaire that included date of birth, sex, race/ethnicity, job title, underlying health conditions, time spent at the retreat, treatment or prophylaxis compliance, and symptoms. The questionnaire was distributed through SurveyGizmo (Widgix, LLC, Boulder, CO, USA), an online vendor with a password-protected website that had an existing Health Insurance Portability and Accountability Act of 1996 business associate agreement with PHSKC. Demographic information was obtained from employee health records for nonrespondents.

HCP met the case definition if they had attended the retreat for any period during September 21–25, 2009, and had laboratory evidence of influenza infection by DFA or real-time RT-PCR from a specimen obtained during September 26–28. All persons meeting the case definition were asked to record symptoms and oral temperatures daily for 2 weeks or until no longer symptomatic. Measured fever was characterized as an oral temperature ≥100.5°F during illness. Those persons who reported a fever but did not measure their temperature or measured an oral temperature <100.5°F were classified as having subjective fever.

Specimen Collection

To examine viral shedding quantitatively, we began sampling of nasal washes on September 30. Self-administered nasal washes were performed every Monday, Wednesday, and Friday until 2 consecutive negative real-time RT-PCR results were documented. Instructions on how to perform a nasal wash were provided in writing and demonstrated to each participant individually. Participants were instructed to quickly instill 5 mL of normal saline without preservatives into 1 nostril, immediately tip their head down, and blow their nose forcefully into a paper cup. Using a 3-mL syringe, participants transferred half of the sample to a plastic specimen vial and half to a container of viral transport media. If <2 mL was obtained, the process was repeated by using the other nostril. Samples were transported on ice to our laboratory on the day they were obtained. Participants not available for sampling on the first day of collection were entered into the study on the subsequent sampling day.

Laboratory Testing

Each nasal wash specimen was tested by rapid culture and real-time RT-PCR. Rapid culture was performed by the PHSKC Laboratory to detect viable virus. In this method, 0.2 mL of specimen was injected into 1 shell vial of R-Mix cells (Diagnostic Hybrids, Athens, OH, USA). The vial was centrifuged for 1 hour at 700 × *g* and incubated for 20–24 hours at 36°C. After incubation, the coverslip was stained with influenza A monoclonal antibody by using immunofluorescence (*6*). Viral load was quantified by real-time RT-PCR at the University of Washington Virology Laboratory as the influenza virus RNA concentration per milliliter of sample (*5*). Nasal wash samples were not quantified by culture.

Statistical Analysis

Questionnaire responses, employee health record data, symptom log, and laboratory data were entered into a spreadsheet and analyzed by using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). Frequencies were calculated for categorical variables, and Fisher exact tests were used to examine associations between categorical variables. Medians were calculated for continuous variables. An accelerated failure time model with a Weibull survival function was used to assess the effect of test type (i.e., real-time RT-PCR or rapid culture), presence of measured or subjective fever, and viral load (of the first available serial sample) on duration of viral shedding. This model accounts for interval censoring present in data and uses a range for shedding duration. This range is based on the fact that initiation of shedding was considered to be the day of symptom onset, and cessation of shedding was considered to range from the collection date of the last positive test result to collection date of the first of 2 consecutive negative test results. Shedding duration was calculated as the number of days between initiation and cessation of shedding (*7*). For participants who showed negative results on the first day of nasal wash sampling, cessation was considered to range from symptom onset to obtaining the first of 2 consecutive negative test results.

To evaluate whether fever was a predictor of viral load, a linear regression model was fit by using viral load as the dependent variable. A p value <0.05 was considered statistically significant in all analyses.

Human Subjects Review

This study was conducted as part of an outbreak investigation by PHSKC in collaboration with Hospital A. The study was classified as nonresearch by the Washington State Institutional Review Board and a CDC human subjects review coordinator.

Results

The retreat occurred from 8:00 AM to 5:00 PM daily for 5 days, with optional evening social activities. Forty-six persons participated in retreat activities. Fourteen persons were facilitators for the retreat, did not attend all of the retreat events, and typically participated for <4 hours/day when attending. The remaining 32 core participants attended all 5 days of the retreat and participated in evening activities. Most core participants also stayed overnight in 1 cabin on Thursday, September 24.

On Friday, September 25, the last day of the retreat, the first retreat participant with documented illness experienced a cough. The following day, this person had fever and was DFA positive for influenza A. By Monday, September 28, active surveillance of the 46 retreat attendees by Hospital A staff identified 20 persons with respiratory symptoms; 19 were core participants and 1 was a facilitator. Influenza A virus was detected by DFA or real-time RT-PCR in 17 symptomatic retreat attendees; all were core participants. All viruses from these 17 persons were confirmed by real-time RT-PCR to be pandemic (H1N1) 2009 virus. Thus, of the 32 core participants at the retreat, 17 (53%) were virus positive; 7 of the 17 had measured fever and cough or sore throat. The other 3 participants with respiratory symptoms were virus negative; all were afebrile.

All HCP who were virus positive were treated with oseltamivir, 75 mg orally 2×/d for 5 days, within 48 hours of symptom onset. In accordance with CDC recommendations at that time, ill HCP were excluded from work for 7 days after symptom onset. Core participants and facilitators who did not become symptomatic or positive for influenza A were administered antiviral chemoprophylaxis (oseltamivir, 75 mg orally, 1×/d for 10 days) and were not excluded from work.

Questionnaires were completed by 45 (98%) of 46 retreat attendees, including 16 of 17 persons who were positive for pandemic (H1N1) 2009 virus infection. Sixteen infected HCP provided serial nasal wash samples and completed a symptom log for the duration of their illness. One virus-infected participant did not complete a questionnaire but did provide serial nasal wash specimens. Another participant who was virus positive completed a questionnaire but did not provide serial nasal wash specimens.

HCP infected with pandemic (H1N1) 2009 virus ranged in age from 26 to 33 years (median 28.5 years). Sixteen (94%) infected HCP were women compared with 67% of core attendees who did not meet the case definition (p = 0.06). Three (18%) infected HCP had an underlying medical condition compared with 1 (7%) of 15 core attendees who did not meet the case definition (p = 0.60). Of those infected HCP who completed the questionnaire, cough, myalgias, and headache were the most commonly reported symptoms (Table), with ≥2 of these symptoms reported by all 16 HCP, and all 3 symptoms reported by 13 (81%) HCP. According to questionnaire data or employee health records of all 17 infected HCP, fever ≥100.5°F was measured by 7 (41%) infected HCP, and an additional 5 (29%) infected HCP reported subjective fever. HCP who reported fever typically documented the fever only during the first 1–2 days of illness, and no one reported measuring an oral temperature ≥100.5°F for >4 days. All HCP reported completing antiviral treatment as prescribed, and no deaths or hospitalizations occurred.

Among 7 HCP who measured a fever ≥100.5°F during their illness, 5 (71%) were virus positive by real-time RT-PCR and 3 (43%) were positive by rapid culture >24 hours after defervescence, when they met 2009 CDC criteria for returning to work. Of the 9 HCP who did not measure a fever ≥100.5°F during their illness and therefore did not meet CDC exclusion criteria, 7 (78%) were positive by real-time RT-PCR; 6 (67%) were also positive by rapid culture at the time of collection of the initial serial nasal wash sample ≥3 days after symptom onset. Samples from 12 (75%) of 16 HCP (95% confidence interval 48%–93%) were positive by real-time RT-PCR, and samples from 9 (56%) of 16 HCP (95% confidence interval 30%–80%) were positive by rapid culture at the time they met 2009 CDC criteria for returning to work (24 hours after defervescence). From the onset of symptoms, the duration of viral shedding

Table. Signs and symptoms among 16 health care personnel infected with pandemic (H1N1) 2009, Seattle, Washington, USA*

Sign or symptom	No. (%) persons
Cough	16 (100)
Myalgia	15 (94)
Headache	15 (94)
Chills	12 (75)
Sore throat	10 (63)
Measured fever†	7 (44)
Subjective fever‡	5 (31)
Diarrhea	3 (19)
Vomiting	3 (19)

*Information on all symptoms was not available for 1 infected person.
†Oral temperature ≥100.5°F during illness.
‡Fever but no measurement of an oral temperature ≥100.5°F during illness.

determined by real-time RT-PCR results ranged from 3 to 13 days compared with 3–10 days by rapid culture results. Among 7 infected HCP, virus was detected by real-time RT-PCR and rapid culture for the same number of days. Among 8 HCP, virus was detected ≈2 days longer by real-time RT-PCR than by rapid culture. In 1 HCP, virus was detected by rapid culture 3 days longer than by real-time RT-PCR.

In our initial model using data from all 16 participants, which included test type (real-time RT-PCR vs. rapid culture), presence of fever (measured and subjective), and viral load as predictors of shedding duration, test type was the only significant variable ($p = 0.02$). Our results indicated no association between fever (measured and subjective) and shedding duration ($p = 0.65$) or between viral load and shedding duration ($p = 0.74$). Our final model, which included only test type as a predictor of shedding duration, indicated that shedding duration was significantly different between rapid culture and real-time RT-PCR (Figure 1). Shedding duration measured by rapid culture was 0.73× higher than shedding duration measured by real-time RT-PCR ($p = 0.02$). Eight of 16 HCP remained positive by rapid culture 4.8 days from symptom onset compared with 6.6 days by real-time RT-PCR.

An overall decrease in viral RNA concentration with time was measured and quantified by real-time RT-PCR (Figure 2). The person with the highest concentration of viral RNA on the first day of serial sampling also had one of the longest shedding durations by real-time RT-PCR. However, viral load was not a significant predictor of shedding duration in our analysis. Measured fever and subjective fever were not correlated with viral load ($p = 0.41$ and 0.12, respectively).

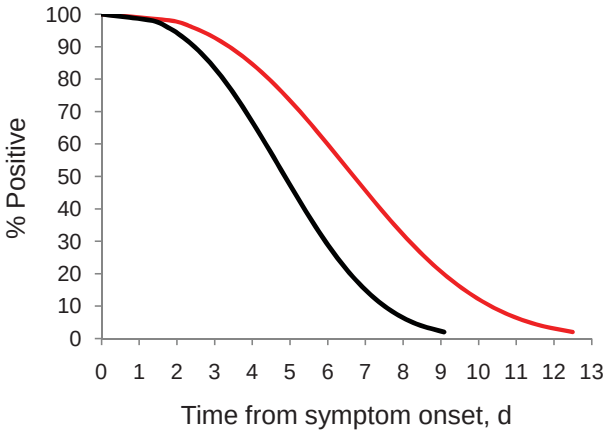

Figure 1. Survival analysis model of pandemic (H1N1) 2009 virus shedding over time among infected health care personnel, Seattle, Washington, USA. Survival curves were modeled on data for 16 persons who became infected with pandemic (H1N1) 2009 virus after attending a work retreat in September 2009. A negative test result by rapid culture (black line) or real-time reverse transcription–PCR (red line) was the event of interest. Shedding duration determined by using real-time reverse transcription–PCR was significantly longer than that determined by rapid culture ($p = 0.02$).

Discussion

We describe a pandemic (H1N1) 2009 outbreak with a high attack rate that involved relatively young, healthy HCP after a hospital-associated retreat. Because of the single source of infection in an enclosed environment, we were able to prospectively monitor study participants to document duration of clinical symptoms and laboratory parameters. Infected HCP often did not report a fever during their illness. Virus infection was documented by real-time RT-PCR and rapid culture for prolonged periods, even though all infected HCP were treated with oseltamivir within 24–48 hours of illness onset. The shedding duration measured by real-time RT-PCR was significantly longer than that measured by rapid culture.

A total of 17 (53%) of 32 participants who attended all 5 days of the retreat became infected with virus. This rate of secondary transmission among core attendees is higher than that of other reports of secondary attack rates, which have been ≤35% (*2,8–10*). The high attack rate we observed might be explained by a susceptible population without immunity to pandemic (H1N1) 2009 virus, active surveillance for infection, or by prolonged close contact among participants, including sharing a cabin. Although viral load of the index case on day 1 of illness was not quantified, the initial viral load (5.03 $\log_{10}$ RNA copies/mL) in this person on day 5 of illness was higher than the day 5 mean and median viral loads reported in other studies (*4,11,12*), which suggested that a high viral load might have contributed to the high attack rate in this outbreak. These findings highlight the potential for rapid spread of influenza among populations with ongoing close contact. In the absence of an available vaccine, handwashing and respiratory hygiene practices (e.g., cough etiquette and exclusion or isolation) should be emphasized in settings where persons might be in prolonged close contact with one another (e.g., hospitals, schools, or shelters).

This study had several limitations. Although all persons received standardized instructions to minimize variation in collection methods, nasal washes were self-collected and had the potential for variability in collection technique. Self-collection of nasal washes might have led to underestimation of presence or quantity of influenza virus. Other studies have demonstrated increased sensitivity with nasal washes, compared with nasal swabs, in respiratory virus detection (*4,13*), and we believe that self-collection of nasal washes likely increased compliance with study procedures.

For logistical reasons, persons were not tested daily, which affected our ability to more precisely define duration

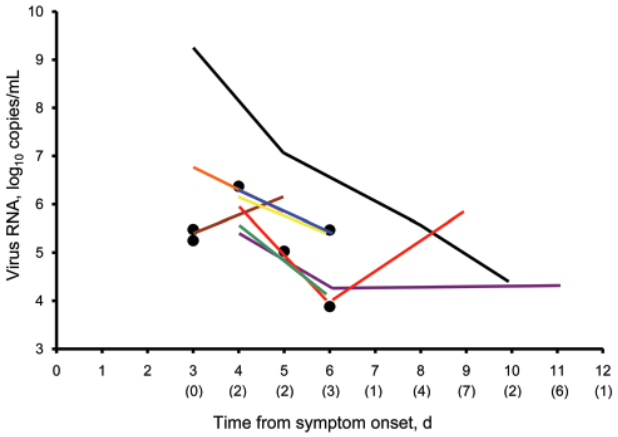

Figure 2. Virus RNA concentrations over time among health care personnel infected with pandemic (H1N1) 2009 virus, Seattle, Washington, USA. Each colored line represents a virus RNA concentration for an infected person tested from symptom onset until the first of 2 consecutive negative results by real-time reverse transcription–PCR (RT-PCR) for pandemic (H1N1) 2009 virus. Persons who had virus detected by real-time RT-PCR only once are indicated by solid circles. The lower detection limit of the real-time RT-PCR was 3 $\log_{10}$ copies/mL. Numbers of persons with virus RNA concentrations below the detection limit for each day after symptom onset are shown in parentheses below the x-axis. Sixteen infected persons were receiving oseltamivir.

of shedding. We chose to use an accelerated failure time model with a Weibull survival function in our analysis because this method accounts for interval censoring. However, our model did not account for the paired nature of the data, in that each sample was tested by rapid culture and real-time RT-PCR. Our unpaired analysis likely led to an overly conservative p value, indicating that a statistically significant p value in our analysis also might have been detected by an analytic technique that accounts for pairing. The small sample size of this study might have decreased the power to detect associations observed in previous studies even if they existed, such as an association between fever and shedding duration or fever and viral load (*12*).

In addition, viral load was not measured on the day of symptom onset in our study but began on the first day that the participant provided serial nasal wash specimens. This delayed measurement might have affected the ability to detect associations between viral load and shedding duration and between fever and viral load. Furthermore, our findings should be considered in light of the fact that all participants were treated with antiviral medication, which might have affected relationships between symptoms, shedding duration, and viral load. Previous research has shown that oseltamivir reduces the duration of symptoms and influenza virus shedding (*14,15*). We did not prospectively ask about the use of fever-reducing medications among infected HCP, and therefore we were unable to definitively assess the potential effect of their use on our findings. Considering that in 6 of 7 HCP who had measured fever during their illness, the fever had resolved at least 36 hours before testing was performed, we do not believe that prospective assessment of the use of fever-reducing medications would have changed our conclusions.

Most characteristics of infection were similar to those of previous reports of pandemic (H1N1) 2009 virus infections (*16–18*), including an outbreak among HCP in Kenya (*8*). Approximately one third of infected HCP in our study did not have measured or subjective fever during their infection. This observation is consistent with those of previous reports (*19,20*). Those HCP who had an oral temperature ≥100.5°F typically documented fever only during the first 1–2 days of infection, although this finding might be explained by oseltamivir treatment that resulted in a shorter duration of symptoms. Fever has been demonstrated in some studies (*12,21*) to correlate with influenza virus shedding. However, we observed that fever was not statistically associated with viral shedding duration (i.e., those HCP who had a fever were not more likely to be positive by real-time RT-PCR or rapid culture longer than those HCP who never had a fever). The association between fever and virus shedding duration might have been modified by antiviral treatment; the small sample size also might have affected the power to detect an association between fever and shedding duration if one existed. In our study, 50% of participants remained positive at 6.6 days by real-time RT-PCR, which is consistent with the average shedding duration reported by Suess et al. (6.6 days) (*4*), but considerably longer than 4.5 days reported by Carrat et al. (*11*).

Most HCP in our study were shedding virus that was detected by real-time RT-PCR and rapid culture after they met CDC criteria for returning to work. Our findings support those of a study that found that persons with influenza infections who shed virus cannot be reliably identified by using fever alone (*11*). These results raise essential considerations regarding exclusion policies for infected HCP. Because febrile and afebrile HCP had similar virologic shedding durations and viral loads, the absence of influenza by real-time RT-PCR or culture might be preferable to the absence of fever as a criterion for HCP who are returning to work in settings where they place others at high risk. A critical caveat is that positive influenza real-time RT-PCR or rapid culture results do not necessarily indicate that the patient is capable of transmitting virus to others. Although reported transmission dynamics of pandemic (H1N1) 2009 virus are similar to those of seasonal influenza viruses (*4*), the generalizability of our findings to seasonal influenza viruses is uncertain.

Our findings, although limited to pandemic (H1N1) 2009 virus infections, suggest that HCP who return to

work after other influenza infections might continue to excrete viable virus and reinforce the need for adherence to infection control measures to prevent transmission in the workplace. All HCP who return to work after influenza infection should practice frequent hand and respiratory hygiene and cough etiquette. Because many HCP were afebrile and others might still be infectious >24 hours after defervescence, our results support the overriding need for influenza vaccination of HCP as the preferred prevention method in health care settings. Further research is needed to determine whether detection of influenza virus and clinical symptoms correlates with infectivity with or without antiviral use.

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Complete Sequence and Molecular Epidemiology of IncK Epidemic Plasmid Encoding *bla*$_{\text{CTX-M-14}}$

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Antimicrobial drug resistance is a global challenge for the 21st century with the emergence of resistant bacterial strains worldwide. Transferable resistance to β-lactam antimicrobial drugs, mediated by production of extended-spectrum β-lactamases (ESBLs), is of particular concern. In 2004, an ESBL-carrying IncK plasmid (pCT) was isolated from cattle in the United Kingdom. The sequence was a 93,629-bp plasmid encoding a single antimicrobial drug resistance gene, *bla*$_{\text{CTX-M-14}}$. From this information, PCRs identifying novel features of pCT were designed and applied to isolates from several countries, showing that the plasmid has disseminated worldwide in bacteria from humans and animals. Complete DNA sequences can be used as a platform to develop rapid epidemiologic tools to identify and trace the spread of plasmids in clinically relevant pathogens, thus facilitating a better understanding of their distribution and ability to transfer between bacteria of humans and animals.

Bacterial plasmids are key vectors of horizontal gene transfer, mediating the mobilization of genetic material from 1 bacterium to another. Their ability to capture DNA and to spread within and between bacterial species by conjugation facilitates the rapid dissemination of potentially beneficial genes through a bacterial population. These genes might alter virulence of the host, confer metabolic benefits, or enable the bacteria to colonize new environments (*1*). Genes that confer resistance to antimicrobial drugs used in human or veterinary medicine are often mobilized on plasmids. One class of resistance genes encode extended-spectrum β-lactamases (ESBLs), which confer resistance against many β-lactam antimicrobial drugs, leading to treatment failures (*2*). Within the past decade, cefotaximase-modifying (CTX-M) β-lactamases have become the most prevalent ESBLs in bacteria isolated throughout the world in hospital and community settings (*3*). More than 85 variants have been identified (www.lahey.org/Studies), mainly in isolates of *Escherichia coli* that cause community-acquired urinary tract infections (*4*). Although clonal expansion events appear to have contributed to the spread of particular CTX-M variants, such as *bla*$_{\text{CTX-M-15}}$ within *E. coli* strain O25:H4-ST131:05 (*5*,*6*), plasmids with the ability to spread efficiently, or epidemic plasmids, also are believed to be responsible for disseminating CTX-M ESBLs (*7*). The ability and frequency with which antimicrobial resistance genes disseminate between bacteria in humans, the environment, and animals is still debated, and the role of plasmids in this movement between ecosystems, including the food chain, is also still contested, despite mounting evidence that it occurs (*8,9*).

CTX-M-14 is the second most frequently identified CTX-M enzyme worldwide (*10*), detected in bacteria isolated from humans, animals, and the environment. CTX-M-14–producing strains show a high level of clonal diversity (*11*,*12*); therefore, dissemination has been attributed to conjugative plasmids rather than to clonal expansion of a bacterial host strain (*13*). In Europe, an association has been suggested between *bla*$_{\text{CTX-M-14}}$ and plasmids of the incompatibility group IncK, or the spread of 1 particular IncK plasmid (*11*,*13*,*14*). In the United

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Kingdom in 2006, Liebana et al. described an ESBL-producing isolate from calves with diarrhea that carried $bla_{CTX\text{-}M\text{-}14}$ on an IncK plasmid, denoted pCT (*15*,*16*). The plasmid spread to unrelated *E. coli* isolates within an index cattle farm and persisted within the environment. In this study, we report the full sequence and analysis of pCT and demonstrate the spread of pCT-like plasmids in animal and human *E. coli* isolates from the United Kingdom, Europe, Australia, and Asia.

Materials and Methods

Bacterial Strains

E. coli C159/11 was isolated from calves on a dairy farm in the United Kingdom in 2004 (*15*,*16*). Investigation and manipulation of the C159/11 plasmid (pCT) was conducted in an *E. coli* DH5α transformant created for the current study. We also investigated 15 CTX-M-14–producing *E. coli* isolates collected during 2006–2009 from cattle feces on farms in different geographic locations in the United Kingdom and 15 *E. coli* clinical isolates from England (P. Hawley, unpub. data), Germany (*17*), Spain (*11,18*), the People's Republic of China (*19*), and Australia (*20*) (Table 1).

Plasmid Extraction and Manipulation

Plasmid pCT was extracted from *E. coli* DH5α transconjugants by using an alkaline sodium dodecyl sulfate Maxi preparation (*21*) and cesium chloride density gradient centrifugation (*22*). Conjugation was by solid mating on a filter (Whatman, Maidstone, UK), by using rifampin-resistant *E. coli* (DH5α) as a recipient and selection of transconjugants on Luria-Bertani agar containing 50 μg/mL rifampin and 8 μg/mL cefotaxime.

Antimicrobial Drug Susceptibility Testing

Susceptibility of C159/11 and pCT transconjugants to a panel of antimicrobial drugs (ampicillin, cefotaxime, cefoxitin, chloramphenicol, ciprofloxacin, naladixic acid, streptomycin, and tetracycline) was determined by using a microtiter broth double dilution method (www.bsac.org.uk/_db/_documents/Chapter_2_Determination_of_MICs_2006.pdf). Susceptibility of *E. coli* DH5α to the antimicrobial drugs tested was also determined.

Table 1. CTX-M-14–producing *Escherichia coli* isolates used in this study*

Origin	Year	Location	Strain/plasmid	Inc type	Source
Cattle	2004	England/Wales	C159/11/ pCT	K	(*15,16*)
Cattle	2006	England/Wales	I779	F, K	NRL
Cattle	2008	England/Wales	I780	F, K	NRL
Cattle	2008	England/Wales	I781	FIA	NRL
Cattle	2009	England/Wales	I782	F	NRL
Cattle	2007	England/Wales	I783	Unknown	NRL
Cattle	2008	England/Wales	I784	Unknown	NRL
Cattle	2008	England/Wales	I785	Unknown	NRL
Cattle	2006	England/Wales	I786	I1-γ	NRL
Cattle	2006	England/Wales	I787	Unknown	NRL
Cattle	2008	England/Wales	I788	Unknown	NRL
Cattle	2008	England/Wales	I789	Unknown	NRL
Cattle	2006	England/Wales	I790	Unknown	NRL
Cattle	2008	England/Wales	I791	F	NRL
Cattle	2008	England/Wales	I792	F	NRL
Cattle	2008	England/Wales	I793	F	NRL
Human	No data	England	L125	Unknown	P. Hawkey, unpub. data
Human	2006	Germany	386	FII	(*17*)
Human	2006	Germany	400	FII	(*17*)
Human	2003–4	Spain	C574	K	(*18*)
Human	2003–4	Spain	C559	K	(*18*)
Human	2003–4	Spain	C567	K	(*18*)
Human	2001–5	Spain	FEC383/ pRYC105	K	(*11*)
Human	2002	Spain	E36/ pRYC110	HI2	(*11*)
Human	1998	People's Republic of China	CH13/ pOZ174	Unknown	(*19*)
Human	2005–7	Australia	JIE 052	B	(*20*)
Human	2005–7	Australia	JIE 081	FII	(*20*)
Human	2005–7	Australia	JIE 084	FII	(*20*)
Human	2005–7	Australia	JIE 088	I1	(*20*)
Human	2005–7	Australia	JIE 182	B	(*20*)
Human	2005–7	Australia	JIE 201	K	(*20*)

*CTX-M, cefotaximase-modifying; NRL, National Reference Laboratory for Enteric Zoonotic Bacteria of Animal Origin of the Veterinary Laboratories Agency, New Haw, UK.

Complete Mucleotide Sequencing of pCT

The plasmid DNA sequence was determined by using a 454/Roche GS FLX analyzer (Roche, Basel, Switzerland). The de novo assembly generated 93631 bases in 7 contigs by using the 454/Roche Newbler assembly program with an N50 of 52,495 bp. The sequence represents improved high-quality draft sequence (*23*) with no discernable misassembles having undergone multiple rounds of computational gap closure. Annotation was completed by using Artemis (Sanger, Cambridge, UK). Further comparative analysis of the DNA sequence used Double ACT and Artemis Comparison Tool (Sanger, Cambridge, UK), DNAstar (Lasergene; Madison, WI, USA) and BLAST (www.ncbi.nlm.nih.gov/guide).

PCR Amplification and Sequencing

Boiled bacterial cell lysates provided template DNA, 1 μL of which was added to PCR ReddyMix Master mixture (Abgene, Epson, UK). Typically, PCR conditions were 30 cycles of 95°C for 30 sec, 51°C for 30 sec, and 72°C for 30 sec. PCR was used to detect $bla_{CTX-M-14}$ and insertion sequences IS*Ecp1* and IS*903* as described (*24*–*26*). All primers used are shown in Table 2. To determine whether the pCT $bla_{CTX-M-14}$ shares a common insertion site with $bla_{CTX-M-14}$ on other plasmids, PCRs were designed to amplify the sequence from $bla_{CTX-M-14}$ into both pCT flanking genes.

Using the pCT sequence, we designed primer pairs to amplify novel regions of pCT for rapid identification of potential pCT-like plasmids in CTX-M-14–producing bacteria (Table 2). PCRs to amplify DNA encoding the putative sigma factor, *pilN* gene, and shufflon recombinase were developed into a multiplex PCR. An additional primer pair was designed to a unique region of pCT and compared with other known sequences for amplification across coding sequences (CDSs) pCT008–pCT009 for further discrimination of pCT-like plasmids. Sequencing of the relaxase gene has been reported to further categorize plasmids within the IncI complex (*11,28*). A modified primer pair was designed for this region (*nikB*) by using sequence data from pCT and other related sequenced plasmids. Resulting amplicons were sequenced by using BigDye Terminator version 3.1 cycle sequencing (Applied Biosystems, Foster City, CA, USA) at the functional genomics laboratory of the University of Birmingham (Birmingham, UK) sequences were aligned by using MEGA 4.0 (*29*) for phylogenetic analysis (*30*). Primers amplifying group 9 bla_{CTX-M} genes were used as a positive control in each instance. The complete DNA sequence of plasmid pCT was assigned GenBank accession no. F868832.

Results

Features of pCT

The $bla_{CTX-M-14}$–carrying plasmid isolated from *E. coli* C159/11 was demonstrated to be conjugative by successful transfer to *E. coli* DH5α by using filter mating and previously determined to be of the incompatibility group IncK (*16*). Analysis of transconjugants showed resistance to β-lactam antimicrobial drugs as the only transferrable resistance phenotype. Whole-plasmid sequencing showed that pCT was 93,629 bp (Figure 1) with an average G+C content of 52.67%. Annotation of the plasmid showed 115 potential protein CDSs, 89 of which were homologous to proteins

Table 2. Primers used for detecting pCT-like regions in plasmids from *Escherichia coli*, United Kingdom, Europe, Australia, and Asia, 2006–2009

Primer	Sequence, 5′ → 3′	Target DNA sequence	Size, bp	pCT binding site	Reference
CTX-M-G9 (F)	ATGGTGACAAAGAGAGTGCAAC	bla_{CTX-M} group 9 variants	876	70259–70280	(*25*)
CTX-M-G9 (R)	TTACAGCCCTTCGGCGATG	bla_{CTX-M} group 9 variants	876	69405–69423	(*25*)
ISEcp1A (F)	GCAGGTCTTTTTCTGCTCC	Insertion sequence ISEcp1	527	71728–71746	(*27*)
ISEcp1B (R)	ATTTCCGGAGCACCGTTTGC	Insertion sequence ISEcp1	527/1,037†	71220–71239	(*27*)
B3A (F)	AACGGCACAATGACGCTGGC	Insertion sequence IS903	887	69913–69932	(*24*)
IS903 (R)	TGTAATCCGGCAGCGTA	Insertion sequence IS903	887	69045–69061	(*24*)
Pseudo (R)	AACATTCGGCCGTTCACAGC	Region downstream of $bla_{CTX-M-14}$	1,636	68644–68663	This study
traK (F)	GGTACCGGCATCGCACAGAA	Region upstream of ISEcp1	1,037	72238–72257	This study
Sigma (F)	ACAGCGTCTTCTCGTATCCA	pCT putative sigma factor	1,289	48590–48609	This study
Sigma (R)	GTTCTTCCAGCTGACGTAAC	pCT putative sigma factor	1,289	47320–47339	This study
pCT *rci* (F)	AAGGTCATCTGCAGGAGT	pCT shufflon recombinase	945	78364–78381	This study
pCT *rci* (R)	GTGTGCGCAGCAACAATA	pCT shufflon recombinase	945	77436–77453	This study
pilN (F)	GACAGGCAGAGAACACCAGA	pCT *pilN* outer membrane protein	627	88267–88286	This study
pilN (R)	ATGCTGTTCCACCTGATGAG	pCT *pilN* outer membrane protein	627	87659–87678	This study
nikB (F)	CGTGCMTGCCGTGARCTT	IncI complex *nikB* relaxase gene	290	33077–33094	This study
nikB (R)	TCCCAGCCATCCWTCACC	IncI complex *nikB* relaxase gene	290	33350–33367	This study
pCT008 (F)	CATTGTATCTATCTTGTGGG	pCT pCT008-pCT009 region	428	3665–3684	This study
pCT009 (R)	GCATTCCAGAAGATGACGTT	pCT pCT008-pCT009 region	428	4074–4093	This study

*pCT, IncK plasmid; CTX-M, cefotaximase-modifying; F, forward primer; R, reverse primer.
†Primer ISEcp1B can be paired with primer ISEcp1A (527 bp) or with primer traK (1,037 bp).

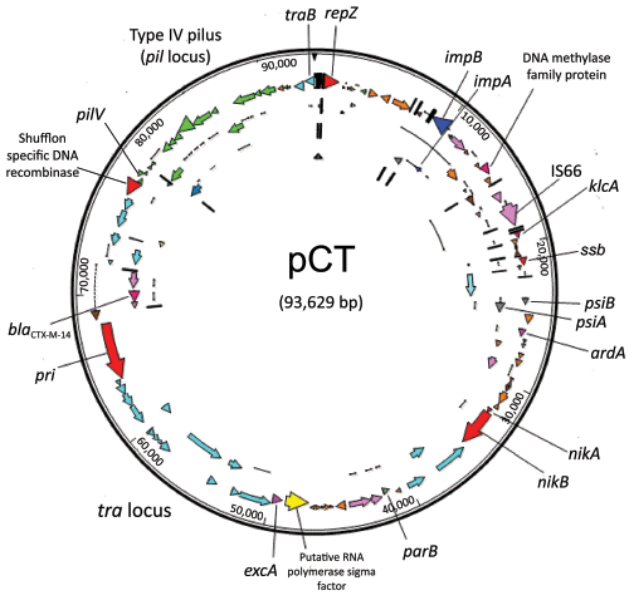

Figure 1. Circular map of plasmid pCT. Open reading frames are color coded as follows: brown, pseudogenes; orange, hypothetic proteins; light pink, insertion sequences; light blue, *tra* locus; green, *pil* locus; dark pink, antimicrobial drug resistance gene; yellow, putative sigma factor; red, replication-associated genes. Arrows show the direction of transcription. pCT, IncK plasmid.

of known function (online Technical Appendix, www.cdc.gov/EID/17/4/645-Techapp.pdf). No genes known to play a role in determining virulence were identified, and sequencing confirmed $bla_{\text{CTX-M-14}}$ as the only antimicrobial drug resistance gene on pCT. The pCT $bla_{\text{CTX-M-14}}$ gene is found between insertion sequences IS*Ecp1* and IS*903* as described (*31*).

Most of the identified putative coding open reading frames are typical for an IncK plasmid backbone (*32*). Two conjugal transfer systems were identified. The first is the more commonly described *tra* operon, which encodes the primary pilus for conjugation transfer. The pCT *tra* locus is analogous to the *tra* operon of R64/ColIb-P9 and is found in a similar conformation, although minor differences existed between the 3 plasmids throughout. The second is the *pil* locus encoding a thin pilus, which is believed to increase conjugation rates in liquid media. The tip of this thin pilus is variable, and the exact nature of the expressed epitope is determined by the orientation and order of *pilV* shufflon components (pCT_094-pCT_098), which can be inverted by the action of the recombinase protein (pCT_093) encoded downstream. Analysis of the pCT plasmid assembly showed that this region was present in multiple forms (data not shown), which is consistent with site-specific recombination mediated by a shufflon recombinase. The pCT shufflon potentially differs from that of the other closely related plasmids (pO113, pO26_vir and pSERB1) because each of these apparently has an inactive shufflon, which can be attributed to the absence of the recombinase or an insertion element within this CDS. The antirestriction and segregation genes on pCT are typical of this type of plasmid.

Comparison of pCT with Other Plasmids

When we compared complete sequences deposited in GenBank of plasmids carrying $bla_{\text{CTX-M}}$ genes with pCT, we found that outside the $bla_{\text{CTX-M}}$ gene those plasmids with different replication mechanisms, such as those within the IncFII group or of the IncN group (pKP96) (*33*), have almost no DNA homology to pCT. The only other $bla_{\text{CTX-M}}$ carrying IncI complex plasmid to be sequenced and deposited in GenBank thus far is a $bla_{\text{CTX-M-3}}$ carrying IncI plasmid pEK204 (EU935740) (*32*). pEK204 shares sequence conservation over ≈60% of the pCT genome, including most of the core IncI complex–related genes for replication or transfer. Further similarities were found in the minimal carriage of resistance genes (Figure 2, panel D).

The pCT genome was also compared with other IncI group sequenced plasmids to identify regions considered core or backbone and to determine novel encoded genes. IncI complex reference plasmids R64 (AP005147) and ColIb-P9 (AB021078) shared 99% identity with 64% of the pCT sequence primarily within genes involved in replication and conjugation (Figure 2, panel C). Other plasmids compared with pCT included R387, the Sanger IncK reference plasmid, which shared a high percentage identity to pCT across IncK-specific core genes (Figure 2, panel B).

We investigated novel genes by identifying regions of the pCT sequence absent from those plasmid sequences with most homology to pCT: pO26_vir (FJ38659), pO113 (AY258503), and the partially sequenced pSERB1 (AY686591), none of which carry $bla_{\text{CTX-M}}$ genes. Both pO26_vir (168,100 bp) and pO113 (165,548 bp) are large plasmids that carry several virulence genes and share 99% homology with 85% and 83% of the pCT genome, respectively (online Technical Appendix Table; Figure 2, panel A). pSERB1 also has high DNA sequence identity (91% of the pSERB1 deposited genome sequence has 99% identity to two thirds of the pCT sequence), however, because this plasmid is deposited in GenBank only as a partial sequence, the total identity cannot be assessed.

Detection of pCT-like Plasmids in Animal and Human Isolates

Fifteen CTX-M-14–producing *E. coli* isolates collected from different cattle farms around the United Kingdom were examined for pCT-like plasmids with a series of PCRs that amplified characteristic regions of pCT. Veterinary

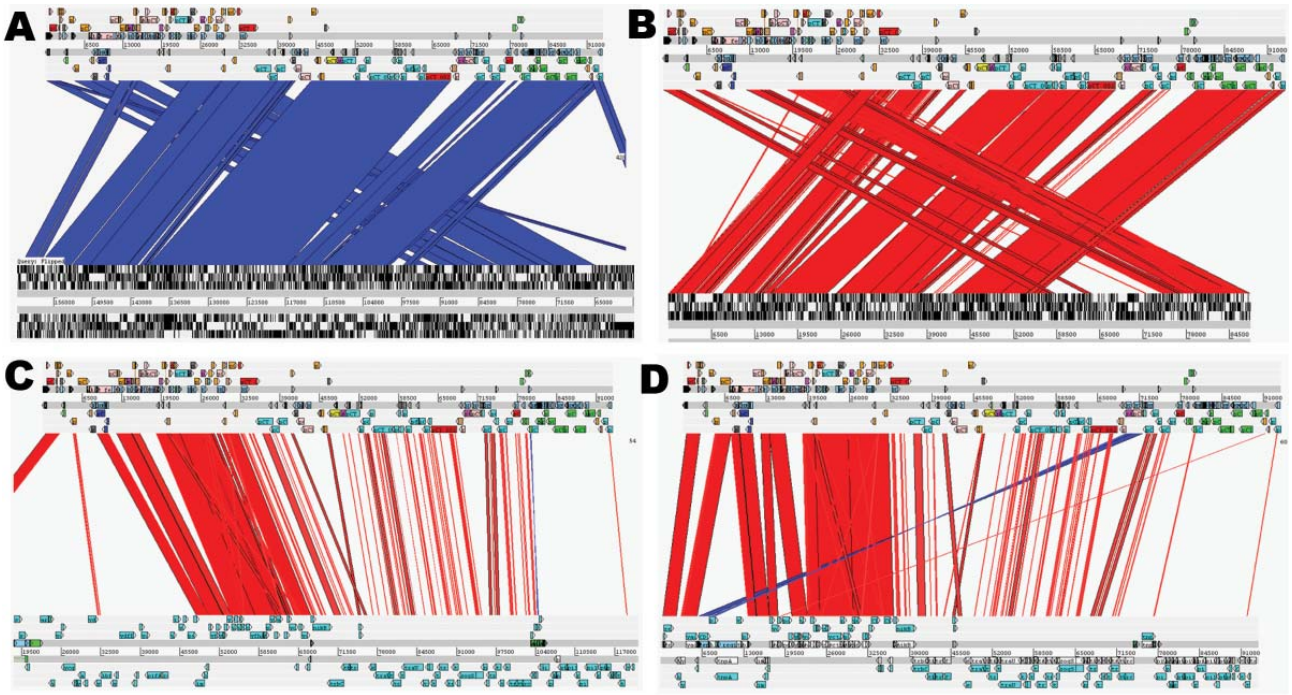

Figure 2. Artemis Comparison Tool (Sanger, Cambridge, UK) comparisons of IncK plasmid (pCT) with other plasmids. Complete DNA sequence plasmid comparisons. Bands of color indicate homology between sequences. Red lines show sequence in the same confirmation; blue lines indicate sequence inversion. The pCT sequence is represented as the top line of each comparison compared with pO26_vir (GenBank accession no. FJ38659) (A); R387 (B); R64 (accession no. AP005147) (C); and pEK204 (accession no. EU935740) (D) on each bottom line.

isolates I779 and I780 were obtained 2 years apart and from different geographic areas in the United Kingdom. These isolates carried plasmids encoding all 6 pCT regions that were investigated ($bla_{CTX-M-14}$, *nikB*, putative sigma factor, *rci*, *pilN*, and pCT008–009) and when compared with pCT had identical flanking regions adjacent to $bla_{CTX-M-14}$. Therefore, these plasmids were deemed pCT-like. Fifteen CTX-M-14–producing *E. coli* human clinical isolates from England, Germany (*17*), Spain (*11,18*), Australia (*20*), and China (*19*) also were examined for pCT (Table 1). No pCT-like plasmids were detected in the isolates from the United Kingdom and Germany. However, pCT-like plasmids were identified in 4 of 5 clinical isolates from Spain (C559; C567; C574; FEC383), 3 of 6 isolates from Australia (JIE 052, JIE 182, JIE 201), and the isolate from China (*E. coli* 8, CH13) because all sequences specific to pCT could be amplified by PCR. pCT-like plasmids have also been shown to be present in other clinical *E. coli* isolates from the United Kingdom by using the same multiplex assay (M. Stokes et al., pers. comm.). Sequencing of amplicons generated during PCR amplification of *nikB* showed pCT-like plasmids had *nikB* sequences with >98% DNA identity to the pCT *nikB* sequence and clustered when these sequences were used to construct a phylogenetic tree (Figure 3). *nikB* sequences from non–pCT-like plasmids clustered further from pCT within the phylogenetic tree (Figure 3). This analysis further supports the hypothesis that pCT has disseminated broadly between bacteria in animal and human ecosystems.

Discussion

We report the complete sequence of a $bla_{CTX-M-14}$–carrying IncK plasmid, pCT, from an *E. coli* isolate from a cattle farm in the United Kingdom (*16*). Within the 115 putative CDs, there is an absence of any genes known to play a role in determining virulence of the host and the absence of any other antimicrobial drug resistance genes except for $bla_{CTX-M-14}$. Therefore, the persistence and spread of pCT cannot be attributed to coselection associated with pressure from non–β-lactam antimicrobial drugs. This finding suggests that pCT persistence and dissemination have been driven by either constant β-lactam exposure or that pCT can remain stable within a population in the absence of any antimicrobial drug selective pressure. There has been much speculation about the role of the type IV pilus and its shufflon found in plasmids and possible role(s) in adhesion of the host bacterium to surfaces and eukaryotic epithelial cells in vitro and in biofilm formation (*34*). The thin pilus might also aid conjugation in a liquid environment by anchoring donor and recipient cells (*35*).

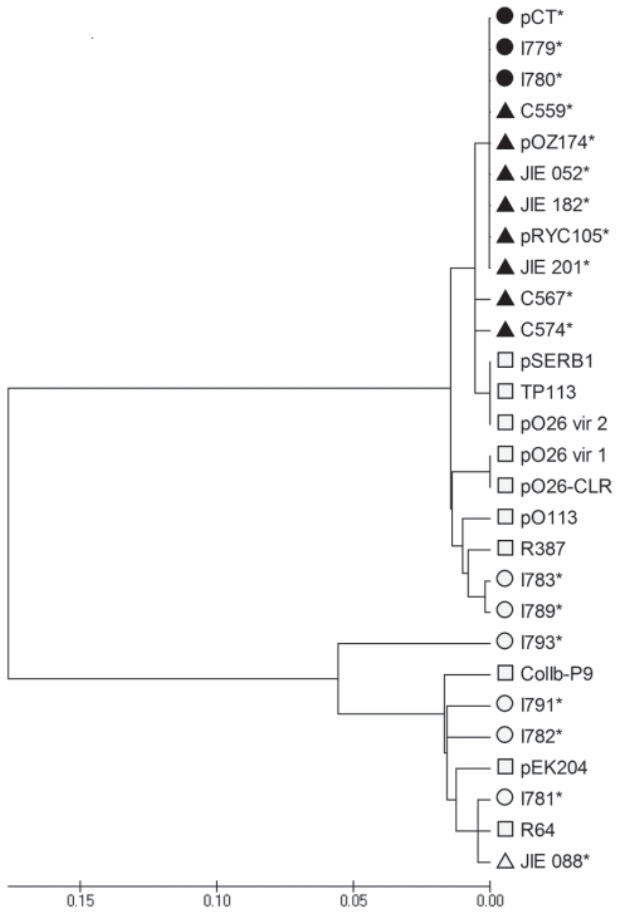

Figure 3. Phylogenetic analysis of *nikB* in IncI complex plasmids from *Escherichia coli*. DNA sequences of *nikB* PCR amplicons and sequences obtained from public resources were aligned and analyzed by using MEGA 4.0 (*19*). A neighbor-joining tree was constructed by using complete deletion modeling and computed by using the maximum composite likelihood method (*30*). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. Circles, *nikB* sequences from plasmids isolated from veterinary isolates from the United Kingdom; triangles, *nikB* sequences of plasmids from *Escherichia coli* isolated from humans; squares, *nikB* sequences of plasmids obtained from GenBank or the Sanger Institute; shaded shapes, plasmids identified as pCT-like by using PCR in this study; asterisks, plasmids encoding $bla_{CTX\text{-}M\text{-}14}$. pCT, IncK plasmid. Scale bar indicates nucleotide substitutions per site.

This system may have played a role in the persistence of *E. coli* C159/11, which originally was found to remain within slurry and on the floor of cow sheds during the longitudinal farm study from which it was identified (*16*). These attributes of the type IV pilus might contribute to the persistence of pCT within bacteria isolated from the UK farm and in animals and humans throughout the world.

Two other features of pCT are of interest. The first, and most notable, is a putative RNA polymerase sigma factor (CDS pCT_066) within the sequence, which shares homology with genes found in only 4 closely related plasmids (pO26vir; pO113, TP113, and pSERB1) and has limited identity to homologue SigB in *Yersinia frederiksenii*. Other weak protein matches show some homology to the extracytoplasmic function sigma factors, small regulatory proteins divergent in sequence to most of the other sigma factors and involved in global gene regulation. Both examples are chromosomally encoded. Although sigma factors of this group have previously been noted on plasmids, scant information has been published about their role or function.

The second feature of interest is that in large stably maintained conjugative plasmids, such as R64, functional large addiction operons such as ParA/B or *kor/mck* usually are present; however, these are lacking in pCT. Despite the apparent lack of stability or persistence genes, pCT has remained stable in a population in the absence of selective pressure for prolonged periods (N.G. Coldham et al., unpub. data).

Comparison of the genome of pCT with other $bla_{CTX\text{-}M}$–encoding plasmids showed no conserved regions outside the β-lactamase gene. Therefore, no single feature of the plasmid backbone appears responsible for the spread of $bla_{CTX\text{-}M}$ genes, and the acquisition of these genes is unlikely to have been a single event. Homology was highest between pCT and 4 plasmids (pO26_vir, pO113, pSERB1, and TP113). pO26_vir was identified in a Shiga toxin–producing *E. coli* strain 026:HII and encodes several virulence genes not found on pCT, including genes for the production of a hydrolase, catalase, and a hemolysin transport protein. pO113 was isolated from another hemolysin-producing EHEC O113:H21 *E. coli* sample from a patient in Australia (*36*). The finding that pCT is most closely related to 2 plasmids that carry an array of virulence genes is of concern because of the potential for recombination between these plasmids, creating mobile elements carrying virulence genes and the $bla_{CTX\text{-}M\text{-}14}$.

The genome sequencing of pCT enabled development of PCRs that amplified discrete regions of the pCT sequence, thereby enabling rapid identification of other pCT-like plasmids that share these loci. pCT-like plasmids were identified in bacteria isolated from 2 other UK farms in 2006 and 2008 and, most recently, from human clinical isolates in the United Kingdom (M. Stokes et al., pers. comm.).

Four human clinical isolates from Spain also carried pCT-like plasmids, with all 6 pCT regions amplified by PCR, which had the same insertion sites for $bla_{CTX\text{-}M\text{-}14}$. These data show the ability of a large conjugative plasmid to transfer between bacteria isolated from humans and animals, facilitating the movement of $bla_{CTX\text{-}M\text{-}14}$ between these niches. Since 2000, when CTX-M-14 was identified in bacteria from Spain, it has become one of the most commonly detected enzymes isolated from human and

animal isolates in Spain (*24,37*). Previous studies conducted in hospitals in Spain examined an association between $bla_{CTX-M-14}$ and IncK plasmids. Valverde et al. (*11*) isolated an IncK plasmid, pRYC105, from many lineages of *E. coli* from community-acquired infections and the environment in different geographic regions of Spain. These authors hypothesized that pRYC105 shared identity with the plasmid isolated in the United Kingdom by Liebana et al. (*16*), and the present study has confirmed this hypothesis by showing that pRYC105 is pCT-like.

Human clinical isolate *E. coli* 8 CH13, described in 2002 and isolated in 1998 from China, contained pOZ174, which encodes $bla_{CTX-M-14}$ (*19*); as with pRYC105, we showed that pOZ174 is pCT like. Furthermore, our data suggest that pCT has persisted since 1998 and is distributed across Europe, Asia, and Australia in diverse *E. coli* lineages isolated from humans and animals. Because CTX-M-14 is the most frequently identified ESBL in Spain and China, further investigation using this molecular test will determine whether pCT is the dominant vector of $bla_{CTX-M-14}$ in these areas and whether pCT has disseminated to other ecosystems. The identical insertion site for $bla_{CTX-M-14}$ in each of the pCT-like plasmids investigated in our study suggests a single capture of this β-lactamase gene onto the plasmid backbone and subsequent spread of the plasmid.

The alignment and analysis of *nikB* from pCT-like plasmids were also used to determine how related the plasmids are and demonstrated sequence identity of >98%. These sequences clustered with pCT within a phylogenetic tree, which indicated less sequence divergence than with other IncI complex non–pCT-like plasmids. Design of the pCT–specific PCRs distributed throughout the plasmid and sequencing of *nikB* amplicons provided a useful and rapid tool in first identifying pCT-like plasmids. Relaxase or *nikB* typing also would provide a suitable locus in recently developed plasmid multilocus sequence typing. These assays can now be used to screen CTX-M-14–producing bacteria for other pCT-like plasmids.

The sequence of pCT enabled an understanding of its backbone and seems to suggest that, apart from plasmid replication and transfer functions, the only known gene that confers a selective advantage on this plasmid is $bla_{CTX-M-14}$. Subsequent PCRs successfully indicated that pCT-like plasmids are distributed over several countries worldwide in bacteria isolated from humans and animals. This approach can be applied to the study of other plasmids of clinical relevance and facilitate better trace analyses of horizontally acquired antimicrobial drug resistance or virulence genes. Additionally, use of this method may lead to identification of new vectors and increase understanding of the interaction among bacteria isolated from humans, animals, and the environment.

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Ms Cottell is a PhD candidate within the Antimicrobial Agents Research Group at the University of Birmingham, Birmingham, UK. Her research focuses on the dissemination of the antimicrobial drug resistance gene $bla_{CTX-M-14}$ on an epidemic plasmid.

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H275Y Mutant Pandemic (H1N1) 2009 Virus in Immunocompromised Patients

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Distinguish clinical characteristics associated with treatment-resistant pandemic (H1N1) 2009
- Analyze outcomes of patients infected with treatment-resistant pandemic (H1N1) 2009
- Identify the rate of H275Y mutation development among patients with pandemic (H1N1) 2009 infection in the current study
- Evaluate the virology of treatment-resistant pandemic (H1N1) 2009.

Editor

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Most oseltamivir-resistant pandemic (H1N1) 2009 viruses have been isolated from immunocompromised patients. To describe the clinical features, treatment, outcomes, and virologic data associated with infection from pandemic (H1N1) 2009 virus with H275Y mutation in immunocompromised patients, we retrospectively identified 49 hematology–oncology patients infected with pandemic (H1N1) 2009 virus. Samples from 33 of those patients were tested for H275Y genotype by allele-specific real-time PCR. Of the 8 patients in whom H275Y mutations was identified, 1 had severe pneumonia; 3 had mild pneumonia with prolonged virus shedding; and 4 had upper respiratory tract infection, of whom 3 had prolonged virus shedding. All patients had received oseltamivir before the H275Y mutation was detected; 1 had received antiviral prophylaxis. Three

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patients excreted resistant virus for >60 days. Emergence of oseltamivir resistance is frequent in immunocompromised patients infected with pandemic (H1N1) 2009 virus and can be associated with a wide range of clinical disease and viral kinetics.

The development of antiviral drug resistance in influenza viruses affects patient care. Concerns for worldwide spread of resistant virus are growing (*1*). Approximately 300 patients with oseltamivir-resistant pandemic (H1N1) 2009 virus have been reported to the World Health Organization, with the complexity of treatment and consequences of infection well described (*2*–*5*). Millions of oseltamivir doses have been stockpiled worldwide, representing one of the major interventions to contain and mitigate the impact of influenza and potentially offer treatment to large numbers of patients (*6*,*7*). The efficacy and cost of pharmacologic interventions to contain oseltamivir-resistant virus are of major concern.

Most resistant pandemic (H1N1) 2009 viruses have been detected in immunocompromised patients who received neuraminidase inhibitors, and all but 1 had the H275Y neuraminidase mutation (*2*). This mutation had already been observed before the pandemic (H1N1) 2009 outbreak, for example, it was detected worldwide in healthy patients who had not received antiviral drugs and were infected with seasonal (H1N1) virus during the 2008–09 influenza season (*8*). Efforts are under way to characterize and detect the H275Y mutation, but data on clinical impact and viral fitness (i.e., replicative capacity in vitro and in vivo that can further be correlated with transmissibility and virulence) associated with this mutation are still needed. We describe in detail the clinical features, treatment, outcomes, and virologic data associated with infection caused by pandemic (H1N1) 2009 virus with H275Y mutation in immunocompromised patients.

Materials and Methods

Hematology–oncology patients who were infected with pandemic (H1N1) 2009 virus and received care at adult and pediatric Seattle Cancer Care Alliance (Seattle, WA, USA) units or clinics during May 1, 2009–April 30, 2010, were identified by using infection control data and laboratory databases. All samples with pandemic (H1N1) 2009 virus detected by in-house real-time reverse transcription–PCR targeting the matrix, and hemagglutinin genes were retrospectively tested by our allele-specific real-time PCR (ASPCR) for H275Y genotype (*9*,*10*). ASPCR uses 2 allele-specific forward primers (wild-type and mutant) and a common reverse primer and probe. Wild-type and mutant genotypes were defined by the difference in PCR cycle threshold values (Δcycle threshold$_{\text{mutant–wild type}}$) between the mutant primer and the wild-type primer amplification curves for the same sample. The ASPCR directed toward the H275Y mutation only was designed and validated in our laboratory, with good correlation demonstrated by pyrosequencing (*9*). ASPCR also can provide an accurate quantitative result of mutant percentage in a mixed population, as described (*11*). Samples were not tested for adamantanes resistance because pandemic (H1N1) 2009 virus was considered to be uniformly resistant to adamantanes.

Specimens collected from inpatients or outpatients were either nasal wash (NW) samples or bronchoalveolar lavage (BAL) samples. NW samples were tested by using multiplex real-time reverse transcription–PCR for respiratory syncytial virus, parainfluenza virus 1–4, human metapneumovirus, adenovirus, bocavirus, coronavirus (OC43, 229E, HKU1, and NL63), and rhinovirus at the same time as influenza A subtyping and influenza B. BAL samples were tested for the same respiratory viruses and for bacterial, mycobacterial, viral, and fungal cultures, fungal PCR, galactomanan, cytomegalovirus by shell vial, respiratory syncytial virus by shell vial, respiratory virus direct immunofluorescence assay, and *Pneumocystis* spp. direct immunofluorescence assay. NW samples were collected by instilling 5 mL of normal saline in each nare and having the patient blow his or her nose directly into a sterile cup. For younger children, suction was used to collect nasal secretions. BAL samples were obtained according to a standardized protocol. Samples were refrigerated within 4 h after collection and transported to the molecular virology laboratory. All available samples were kept frozen at –80°C for up to 8 months after initial clinical testing. Total nucleic acid was extracted as described (*12*). Institutional review board approval was obtained from our institutional committee. A chart review of all patients with mutant H275Y virus was retrospectively performed by using standardized case record forms.

Results

ASPCR Results

We identified 49 adult and pediatric hematology–oncology or hemopoietic cell transplant (HCT) patients who were infected by pandemic (H1N1) 2009 virus during May 1, 2009–April 30, 2010. Of these patients, 16 had no specimen available for genotyping of position H275 by ASPCR because the initial diagnostic test was performed in another laboratory (12 patients), no residual sample was available (3 patients), or the viral load was too low to genotype (1 patient). For 33 patients, at least their first sample, obtained before treatment, was available for genotyping. One of the 33 first samples collected had the H275Y mutation. For 17 patients, repeat samples were obtained for clinical reasons, but only 12 patients had sufficient viral load for genotyping. The H275Y mutation

developed in 7 (58%) patients. The H275Y mutation was identified in 8 patients; 3 of these patients (patients 1, 2, and 6) have been described (*5*,*13*).

Clinical Characteristics

Five of the 8 patients with H275Y mutation had undergone allogenic HCT or were receiving conditioning for future allogenic HCT (Table 1). Two patients had malignancies (acute lymphoblastic leukemia and osteosarcoma), and 1 had aplastic anemia. Four were children and 4 were adults. Three patients had severe lymphopenia, with lymphocyte counts persistently <200 × 10^3 cells/L. Clinical characteristics are presented in Table 2; viral kinetics and treatment are presented in the Figure for each patient. No patients had contact with another infected patient in the hospital or the clinic, and no evidence was found of nosocomial transmission of resistant strains.

Severe Pneumonia

Severe pneumonia followed by acute respiratory distress syndrome developed in 1 of the 8 patients (patient 1). This patient was initially treated with oseltamivir for 4 days; then treatment was changed to intravenous peramivir because of the patient's inability to tolerate oral therapy. Just before initiation of peramivir, a BAL sample showed the absence of H275Y mutation in the viral population that were present in the lung but no concomitant NW sample was available for testing. After 7 days of intravenous peramivir, an NW sample showed H275Y mutation in 100% of the viral population. This patient subsequently received triple-combination antiviral drug therapy (i.e., oseltamivir, rimantadine, and oral ribavirin) while awaiting intravenous zanamivir that was administered during days 18–25. The patient died of severe pneumonia and multiorgan failure after several days of mechanical ventilation. Autopsy showed necrotizing pancreatitis with bilateral pulmonary consolidation, pulmonary hemorrhage, diffuse alveolar damage, and patchy fibrosis. Pandemic (H1N1) 2009 virus was proven by PCR in the NW sampled at autopsy but not in the lung tissue or pancreas. Influenza viruses detected in the NW samples were completely wild-type at autopsy.

Mild Lower Respiratory Tract Infection with Prolonged Shedding

In 3 patients (patients 2–4), lower respiratory tract infection quickly developed, but the patients recovered without complications. Two of these patients initially were treated with oseltamivir alone, and 1 was treated with oseltamivir and rimantadine. Viral H275Y mutation was detected at days 23, 8, and 17 for patients 2, 3, and 4, respectively. All 3 patients had prolonged mild upper respiratory tract symptoms, consisting mainly of rhinorrhea and dry cough, accompanying a variable duration of viral shedding. Influenza virus was detected in the NW sample for 93, 8, and 47 days in patients 2, 3, and 4, respectively. Patient 2 had shedding of fully resistant viral population documented for at least 26 days after antiviral drug therapy was stopped. Patient 3 recovered after 17 days of oseltamivir therapy, even though a small percentage of H275Y mutations (10%) were present in his NW sample at day 8. In patient 4, the percentage of H275Y mutants declined slightly after treatment was stopped. Two of these 3 patients had substantial concurrent pathogens (*Pneumocystis jiroveci* and *Aspergillus fumigatus*) in BAL samples at the same time as pandemic (H1N1) 2009 virus infection, making the diagnosis of pandemic (H1N1) 2009–related pneumonia less certain.

Upper Respiratory Tract Infection with Prolonged Shedding

Three patients (patients 5–7) had symptoms of only upper respiratory tract disease. None had hypoxemia or infiltrate on chest radiograph, but all 3 had a cough and 2 had fever for 24 hours. They all had profound lymphopenia (0–366 × 10^3 cells/L) and 1 had graft-versus-host disease. Two patients were initially treated with oseltamivir alone and 1 with oseltamivir and rimantadine. One hundred percent mutant virus developed in patients 5–7; these patients shed influenza virus for 65, 75, and 12 days, respectively. Patient 5 maintained a 100% mutant viral population while receiving peramivir and continued to maintain this percentage while on prolonged oseltamivir therapy. Patient 6 remained infected with a fully resistant viral population many days after oseltamivir was stopped. Patient 7 improved rapidly, but because of underlying lung disease, PCR was repeated on day 12 and showed continual viral shedding with a low viral load. No other viral testing was performed, and this patient recovered completely with a second 5-day course of oseltamivir.

Prophylaxis

One young patient (patient 8) had 85% H275Y mutant viral population detected in the first NW sample obtained on day 2 of illness. This patient had been in contact with 2 family members who had documented influenza infection but did not receive antiviral drug therapy. The patient then received oseltamivir prophylaxis (45 mg 2×/d) for 10 days; 3 days after prophylaxis was completed symptoms developed, including fever, chills and sore throat. The patient was treated with 10 days of oseltamivir and had a mild course of disease with rapid clinical resolution.

Initial Therapy

In all patients, except patient 8, virus was fully wild-type when the initial specimen was collected. All patients had received antiviral drug therapy before the H275Y

Table 1. Demographic characteristics and underlying conditions in 8 patients with H275Y mutation of pandemic (H1N1) 2009 virus, Seattle Cancer Care Alliance, Seattle, Washington, USA, May 1, 2009–April 30, 2010*

Patient no.	Age, y/sex	BMI	Underlying disease	State of disease	HCT	Recent immunosuppressive therapy	Concurrent illness	Lymphocytes, $\times 10^3$ cells/L
1†	51/M	20.5	AML	Remission	2 y post–allo-HCT	MMF, tacrolimus	GVHD, renal failure	110
2†	47/F	22.9	AML	Relapse	3 y post–allo-HCT	Chemotherapy 1 mo before influenza dx and 1 d after-dx	None	540
3	50/M	23.3	CML	Remission	9 mo post–allo-HCT	TBI, cyclophosphamide, tacrolimus	GVHD	1,470
4	7/F	18.9	ALL	Relapse	NA	Chemotherapy 2 d after influenza diagnosis; prednisone 1.5 mg/kg/d	None	1,440
5	34/F	38.8	Hogkin lymphoma	Refractory	2 d pre–allo-HCT	TBI, fludarabine, cyclophosphamide	Renal failure	0
6†	17/M	19.5	ALL (T cell)	Remission	3 wk post–allo-HCT	TBI, fludarabine, cyclophosphamide, MMF, CSA, prednisone 0.5 mg/kg/d	CMV, GVHD	154
7	17/M	22.4	Osteosarcoma	Relapse	NA	Chemotherapy 1 d before influenza diagnosis	Lung metastasis, thoracotomy, restrictive syndrome	366
8	8/F	14.5	Aplastic anemia	Treatment	NA	ATG, CSA maintenance	None	1,145

*BMI, body mass index; HCT, hemopoietic cell transplant; AML, acute myeloblastic leukemia; allo, allogenic; MMF, mycophenolate mofetil; GVHD, graft-versus-host disease; NA, not applicable; CML, chronic myeloblastic leukemia; TBI, total body irradiation; ALL, acute lymphoblastic leukemia; CSA, cyclosporine; CMV, cytomegalovirus; ATG, antithymocyte globuline.
†Patients previously reported in references (*5,13*).

mutation was detected. One patient had received oseltamivir prophylaxis, 4 had received only oseltamivir treatment, 2 had received combination oseltamivir/rimantadine therapy, and 1 had received oseltamivir followed by intravenous peramivir.

Virologic Data

In 3 of the 8 patients (patients 4–6), viral loads increased after H275Y viruses were detected. In 2 patients (patients 2 and 4), viral loads declined after start of either intravenous or inhaled zanamivir. In 1 patient, viral load declined during 17 days of oseltamivir therapy, even though 10% of the viral population was documented to be H275Y mutants. Five of the 8 patients were found at some time to have mixed, wild-type and mutant, populations. We did not identify any discrepancy between BAL sample and NW sample genotyping results, but both types of samples were collected in close proximity (within 2–4 days) in only 3 patients. Wild-type virus was detected in 2 patients in paired NW and BAL samples; mutant virus was detected in paired NW and BAL samples in 1 patient. The 3 patients with the highest initial viral load (>6 $\log_{10}$ copies/reaction) had a substantial percentage of H275Y mutants at days 8, 9, and 11 (range 35%–100%). Patients with lower initial viral loads were not tested a second time until 12–23 days later. These long intervals do not enable us to compare the timing of resistance emergence according to initial viral load.

Discussion

During the pandemic (H1N1) 2009 outbreak, neuraminidase-resistant viruses emerged rapidly in immunocompromised patients. Our retrospective analysis suggests a high rate of oseltamivir resistance conferred by the H275Y mutation in treated immunocompromised patients. The rate of mutation development ranged from 8 (16%) of 49 patients, with all infected patients as denominator, to 7 (58%) of 12 on the basis of samples obtained after antiviral drug therapy began. Two other studies have reported similar rates. One study from Scotland reported a 50% rate (5/10) of H275Y mutation in immunocompromised patients with samples available after oseltamivir therapy began (*14*). The other study, in Australia, reported a 13.3% rate (4/30) of H275Y mutation in treated immunocompromised patients (not all of them tested) or 57% rate (4/7) in only treated patients for whom samples were available after treatment began (*15*).

The 8 patients in our study who had H275Y mutant virus demonstrated a wide range of clinical disease, from benign upper respiratory tract symptoms to severe and fatal respiratory insufficiency. Whether the H275Y mutation is associated with higher rates of death or severe disease is unclear. The small number of immunocompromised patients infected with resistant pandemic (H1N1) 2009 virus did not enable us to compare them with patients infected with sensitive pandemic (H1N1) 2009 virus. However, our case

Table 2. Clinical characteristics and outcomes of 8 patients with H275Y mutation of pandemic (H1N1) 2009 virus, Seattle Cancer Care Alliance, Seattle, Washington, USA, May 1, 2009–April 30, 2010*

Patient no.	Symptoms of URTI	Signs of LRTI	Radiology results	Antiviral drug therapy before resistance	Co-pathogens	Outcome
1	24 h before diagnosis: congestion, headache	Hypoxemia; positive BAL result on d 5	Bilateral ground glass opacity	Oseltamivir 150 mg 2×/d followed by peramivir	None	Death related to influenza
2	48 h before diagnosis: congestion, wet cough, sore throat, fever (24 h)	Hypoxemia; positive BAL result on d 25	Bilateral ground glass opacity	Oseltamivir 150 mg 2×/d + rimantadine 100 mg 2×/d	*Pneumocystis* spp. (DFA + in BAL)	Alive
3	5 d before diagnosis: fever (24 h), wet cough	Hypoxemia; positive BAL result on d 2	Multiple nodules with halo sign	Oseltamivir 150 mg 2×/d	Aspergillus (PCR and GM result positive in BAL sample); *Staphylococcus aureus*; PIV3	Alive
4	<24 h before diagnosis: fever (5 d), cough	Hypoxemia	Bilateral infiltrates	Oseltamivir 2 mg/kg 2×/d	None	Alive
5	<24 h before diagnosis: cough, rhinorrhea, congestion	No	Bronchial thickening	Oseltamivir 150 mg 2×/d	None	Alive
6	<24 h before diagnosis: rhinorrhea, fever (24 h), cough	No	None	Oseltamivir 150 mg 2×/d	None	Alive
7	24 h before diagnosis: sore throat, fever (24 h), cough	No	CXR stable	Oseltamivir 75 mg 2×/d + rimantadine 100 mg 2×/d	Rhinovirus (PCR result positive in NW sample)	Alive
8	24 h before diagnosis: sore throat, fever (24 h), chills	No	CXR normal	Prophylaxis: oseltamivir 45 mg 2×/d for 10 d, ended 3 d before influenza diagnosis	None	Alive

*URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; BAL, bronchoalveolar lavage; DFA, direct immunofluorescence assay; GM, galactomanane; PIV3, parainfluenza virus 3; NW, nasal wash; CXR, chest radiograph.

series highlights the rapid emergence of resistant viruses in the context of mild to severe influenza disease. In 7 of the 8 cases in our study, resistance was not associated with long-term consequences and was not even suspected in 4 of the 7 cases. This observation could suggest that resistant viruses are not more virulent than wild-type viruses.

Influenza resistance kinetics as provided in this study by the percentage of H275Y mutants enable a better determination of the timing of emergence of resistant virus and the possible subsequent clearance of this resistant viral population. Our results suggest that elevated viral loads seen early in disease might be associated with more rapid emergence of resistance, but more complete and regular testing after the initial sample is necessary to confirm that hypothesis.

In this case series, some patients conserved the resistant viral population after antiviral pressure removal, whereas another patient (patient 1) cleared his resistant viral population to recover a fully wild-type virus. Similarly, the H275Y mutation described in another case report disappeared after antiviral drug therapy was completed, while others showed conserved resistant viral population (*14*,*16*). Those 2 different evolutions of mutant viral population suggest the possibility of quasispecies with different fitness. The H275Y neuraminidase mutation had affected in vitro viral fitness when incorporated in seasonal (H1N1) virus, but data looking at 2008–09 seasonal (H1N1) virus showed that the circulating H275Y mutant strain had recovered its fitness, possibly explaining its sustainable transmission (*17*). Recently, permissive secondary mutations in the neuraminidase gene leading to fitness recovery have been proposed in seasonal (H1N1) viruses (*18*). Substitutions V234M and R222Q buffer deficiencies in neuraminidase folding or stability caused by H275Y and simultaneously allow the virus to keep its neuraminidase/hemagglutinin balance, which is implicated in viral fitness (*19*). Further molecular and in vitro analysis of viral strains from patients who cleared and did not clear the mutant viral population might provide more input about permissive secondary mutations in pandemic (H1N1) 2009 virus.

We were not able to provide information about whether an H275Y mutant viral population can develop in the upper respiratory tract and not in the lower respiratory tract or the converse scenario. The limited data we described on paired NW and BAL samples suggest that the upper and lower respiratory tracts are likely to be infected with the same viral H275Y genotype. Others have shown that quasispecies harboring the D222G hemagglutinin mutation that confers improved pneumocyte receptors binding could be found specifically or in larger proportions in endotracheal aspirates than in paired nasopharyngeal aspirates (*20*). Analysis of D222G mutant virus has not yet been reported in immunocompromised patients, a condition in which mutations develop more readily.

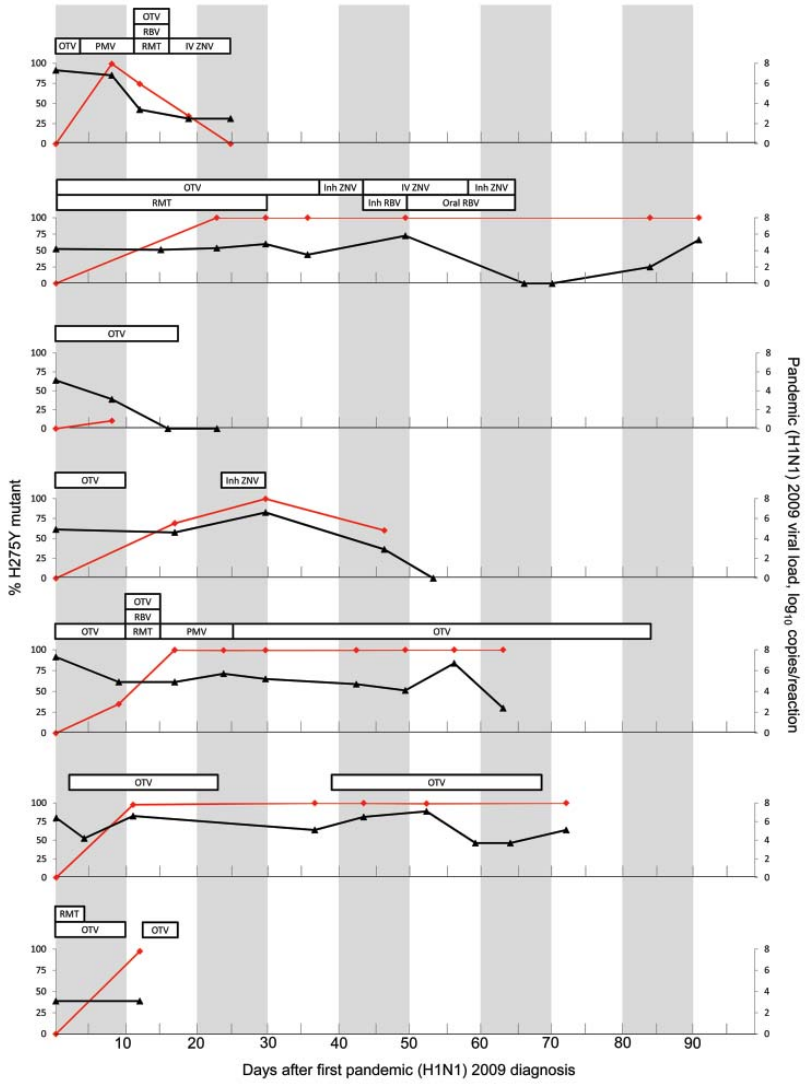

Figure. Viral kinetics in nasal washes and treatment data of 7 patients (patient nos. 1–7 shown top to bottom) with H275Y mutation of pandemic (H1N1) 2009 virus, Seattle Cancer Care Alliance, Seattle, Washington, USA, May 1, 2009–April 30, 2010. Black lines, pandemic (H1N1) 2009 viral load; red line, % H275Y mutant. OTV, oseltamivir; RBV, ribavirin; RTM, rimantadine; ZNV, zanamivir; PMV, peramivir.

We have demonstrated that the H275Y mutation can develop in immunocompromised patients under different antiviral drug treatment regimens. Oseltamivir is the antiviral agent most associated with the emergence of H275Y. Peramivir selects for the H275Y mutation in seasonal (H1N1) by successive passages in vitro, but data have not clearly confirmed this observation in vivo (*21*). One patient in our study had received oseltamivir and peramivir before resistance detection. We believe that if peramivir was not the drug that selected the initial mutation, it did not prevent establishment of dominant H275Y virus population (*13*). Because peramivir is rarely used as frontline therapy, data to support this hypothesis are likely to be difficult to obtain. Combination therapy with oseltamivir and rimantadine did not prevent development of H275Y mutation in 2 of the patients in our study. The issue of antiviral drug resistance in immunocompromised patients makes determination of optimal initial therapy necessary. Inhaled zanamivir is often contraindicated because the patients may have respiratory failure or underlying lung disease, and novel agents (e.g., DAS181) are not yet available. Triple-combination therapy (i.e., oseltamivir, rimantadine, and oral ribavirin) potentially could reduce emergence of resistance, but more data are needed to support this hypothesis (*22*,*23*).

This case series has some limitations. It does not provide a totally accurate incidence of resistance in immunocompromised patients. Some cases of pandemic (H1N1) 2009 might have been missed, particularly if symptoms were mild or if the patient was not identified by infection control surveillance. Also, repeat testing was performed in only 17 patients, in whom 5 viral loads were too low to genotype. Systematic testing of infected patients at 5 or 6 days after initial pandemic (H1N1) 2009 diagnosis might have caught even more minor mutant viral

populations. Our ASPCR was aimed only at detection of the H275Y mutation. Presence of other rare mutations as the I223R could have influenced our results (*24*,*25*). However, our data highlight the need for surveillance and clinical testing for resistant mutations in immunocompromised patients by using sensitive and rapid molecular diagnostic tests.

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Mumps Complications and Effects of Mumps Vaccination, England and Wales, 2002–2006

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe the clinical presentation and short- and long-term complications associated with mumps infection
- Describe the attack rate of mumps in the United Kingdom epidemic of 2004–2005
- Identify the most common mumps complications seen in hospitalized children in the mumps epidemic
- Compare hospitalization and complication rates for mumps among MMR-vaccinated and unvaccinated children.

Editor

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We analyzed data from hospital admissions and enhanced mumps surveillance to assess mumps complications during the largest mumps outbreak in England and Wales, 2004–2005, and their association with mumps vaccination. When compared with nonoutbreak periods, the outbreak was associated with a clear increase in hospitalized patients with orchitis, meningitis, and pancreatitis. Routine mumps surveillance and hospital data showed that 6.1% of estimated mumps patients were hospitalized, 4.4% had orchitis, 0.35% meningitis, and 0.33% pancreatitis. Enhanced surveillance data showed 2.9% of mumps patients were hospitalized, 6.1% had orchitis, 0.3% had meningitis, and 0.25% had pancreatitis. Risk was reduced for hospitalization (odds ratio [OR] 0.54, 95% confidence interval [CI] 0.43–0.68), mumps orchitis (OR 0.72, 95% CI 0.56–0.93) and mumps meningitis (OR 0.28, 95% CI 0.14–0.56) when patient had received 1 dose of measles, mumps, and rubella vaccine. The protective effect of vaccination on disease severity is critical in assessing the total effects of current and future mumps control strategies.

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Mumps is an acute viral infection that is asymptomatic in ≈30% of children (*1*). Symptoms and signs include fever, headache, and swelling of the parotid glands, which may be unilateral or bilateral. Complications of mumps

include orchitis, aseptic meningitis, oophoritis, pancreatitis, and encephalitis (*2*–*4*). Long-term complications include unilateral sensorineural deafness in children (*5*). To date, reported data on mumps complications are based on studies conducted mainly during the prevaccine era. Mumps vaccination was introduced into the UK immunization program as a single-dose mumps, measles, and rubella vaccine (MMR) for children 12 to 15 months of age in October 1988. The first vaccines contained the Urabe strain but this was changed to the Jeryl-Lynn strain in 1992 because of an unacceptable risk for aseptic meningitis (*1*). In 1996, to provide additional protection against all 3 infections, a second dose was added to the schedule. In the first decade after the MMR was introduced, rates of reported and confirmed mumps virus infection fell to extremely low levels in the United Kingdom. For persons born in the first 10 years of the program (1988–1998), vaccination coverage reached >90% for the first dose and ≈75% for the second dose of MMR by 5 years of age (*6*). Vaccine effectiveness in the UK has been estimated to be 87.8% for 1 dose and 94.6% for 2 doses of vaccine (*7*).

Since 1998, however, several mumps outbreaks have occurred in adolescents and young adults; these culminated in a national epidemic, mainly affecting university students, in 2004 and 2005. Clinical notifications of mumps increased from 4,203 in 2003 to 16,436 in 2004. The attack rate by birth rate was highest in those born between 1983 and 1986, with a rate of infection ranging from 140 to 170 per 100,000 population (*8*). Persons in this cohort were not offered routine childhood MMR and avoided mumps exposure because of high coverage in younger children. The rate of infection in persons born after 1988, and eligible to receive MMR, was substantially lower, and only 2.4% occurred in age groups eligible for 2 doses of MMR (*8*). Recent mumps surveillance data in England and Wales are showing an increase in the proportion of mumps cases in cohorts who should have received the 2-dose MMR (*9*). Two-dose MMR coverage in these cohorts has been estimated as ≈75% (*10*). Resurgences of mumps in vaccinated populations (including those who received 2-dose MMR) have been described in educational settings in other countries (*11*–*15*). Declining protection over time, and possible antigenic differences between the vaccine and outbreak strains, have been suggested as contributory factors (*7*,*16*,*17*). In the absence of natural boosting, therefore, future mumps epidemics may be unavoidable in vaccinated populations living in crowded, semiclosed settings such as colleges (*18*).

Because mumps is more severe in adults, increasing numbers of mumps cases in young adults in the postvaccine era could be expected to lead to a high rate of complications. A better understanding of mumps complications in vaccinated persons will therefore be essential in developing appropriate strategies to control mumps. We investigated hospitalizations associated with the mumps epidemic in England and Wales in 2004–2005 and used enhanced surveillance to compare the rate of complications among patients with confirmed mumps cases by age and vaccination status.

Methods

We analyzed hospital episode, enhanced surveillance data, and clinical and laboratory surveillance data on mumps cases with onset or admission from April 1, 2002, through March 31, 2006, covering the period of the mumps outbreak in 2004–2005. When no onset date was available, the date of the sample or report was used.

Hospital Episode Statistics

Hospital episode statistics (HES) capture all admissions to National Health Service (NHS) hospitals in England and Wales. The diagnoses recorded at the time of discharge are coded by using the International Classification of Diseases, 10th edition (ICD-10), and entered in any of 13 fields. A minimum dataset was extracted for all admissions with any of the following codes: B26 (mumps), N45 (orchitis and epididymitis), A87 (viral meningitis), N70 (oophoritis), and K85 (acute pancreatitis) (*19*). The anonymized HES identification field, generated from the NHS number, local patient identifier, postcode, sex, and date of birth, was used to link episodes from the same person admitted over the period (*20*).

Enhanced Surveillance

In England and Wales, clinicians who diagnose mumps are required by statute to notify the proper officer for the local authority, usually a consultant in health protection. Since 1995, all notified cases of mumps have been monitored by offering oral fluid testing for immunoglobulin (Ig) M at the Centre for Infections, Health Protection Agency. A high proportion of cases are tested (50%–80%), and thus cases confirmed by testing for IgM in oral fluid provide data on the incidence of mumps (*1*). Vaccination history is requested on the sample-testing form for the oral fluid sample.

All patients with confirmed cases were then followed up by sending an enhanced surveillance form to the general practitioner (directly or through the local health protection unit) requesting further information. Information on complications, whether the mumps case-patient was hospitalized, and the receipt of MMR (or other mumps virus–containing vaccines) was confirmed. Those with no record of vaccination shown on the sample request form and in the general practitioner records (as noted on the returned enhanced surveillance forms) were classified as unvaccinated. Complications were recorded in free

text, which was searched and recoded specifically for any mention of orchitis, meningitis, pancreatitis, and oophoritis.

Estimating Total Mumps Cases from Laboratory-confirmed Mumps

Because of the high proportion of patients with notified cases that are tested by oral fluid, results for laboratory-confirmed mumps are thought to provide fairly complete estimates of clinically diagnosed mumps incidence. In 2005, however, during the peak of the mumps outbreak, mumps oral fluid testing was temporarily suspended in those born from 1981 through 1986. Therefore, to provide a better estimate of true incidence of clinically diagnosed mumps in 2005, we extracted the number of patients with clinically notified cases of mumps born during 1981–1986 from notifications of infectious diseases. In view of the high positive predictive value of clinical diagnosis in this age group and period, the number of clinically diagnosed patients with notified cases was then used as the total estimated denominator instead of laboratory-confirmed mumps cases.

Statistical Analysis

Logistic regression analysis was used to assess the relation between hospitalization, mumps complications, and vaccination status in the enhanced surveillance data. The model was adjusted for age and sex, except in the model with mumps orchitis in which only male patients were included, and adjustment was made only for age. The age variable was included as a continuous variable by using polynomials up to the fifth degree to allow for nonlinearity of age. This option is an alternative to using a large number of age categories (which would give similar results). Odds ratios (ORs) with 95% confidence intervals (CIs) were determined; $p<0.05$ was considered significant. All statistical analysis was performed in Stata version 11 (StataCorp, College Station, TX, USA).

Results

The total estimated number of mumps case-patients in England and Wales from April 1, 2002, through March 31, 2006, obtained by combining data from laboratory diagnosis and notifications (for those born between 1981 and 1986 in 2005 only), was 43,344 (23,246 male). A total of 2,647 mumps case-patients were hospitalized from April 2002 through March 2006 (Table 1). Hospitalized mumps case-patients, including those with a code for orchitis (996 patients), meningitis (154 patients), pancreatitis (146 patients), or none of these complications (1,418 patients) showed a clear increase during the outbreak (Figure). No hospitalized mumps case-patients also had been given a code for oophoritis. Most of these mumps complications were attributable to the mumps outbreak because episodes with these codes were negligible before the start of the outbreak period. Of mumps complications in hospitalized patients, most (81% of those with orchitis, 76% with meningitis, and 78% with pancreatitis) arose in those born from 1980 to 1989 (Table 1).

Therefore, the estimated rate of hospitalization was 6.1% (2,647/43,344) overall. The hospitalization rate was 4.4% (996/22,686) for mumps orchitis, 0.35% (154/43,344) for mumps meningitis and 0.33% (146/43,344) for mumps pancreatitis. When results are stratified by year of birth to those born before 1980, those born from 1980 through 1989, and those born from 1990 through 2006, the estimated complication rate for hospitalized mumps orchitis is lower in younger age groups than in older age groups (Table 1). Although rates of mumps meningitis and mumps pancreatitis in younger cohorts are lower, the pattern is less clear because of the smaller numbers involved. The rate of hospitalized mumps not coded with any of the main complications was lowest in the 1980–1989 cohort.

From April 2002 through March 2006, a total of 28,280 laboratory-confirmed mumps cases occurred. For 15,524 (55%) case-patients, the enhanced surveillance form was completed and returned. The response rate was higher in younger age cohorts. For those born pre-1980, the response rate was 52% (2,298/4,455), compared with 55% (10,865/19,763) for those born from 1980 through1989 and 68% (2,554/3,737) for those born from 1990 through 2006. For those born since 1990, the response rate was higher for confirmed case-patients listed as vaccinated on the sample request form (1765/2,189 [81%]) than for those with no vaccination details (789/1,548 [51%]).

Of 15,524 confirmed mumps case-patients whose enhanced surveillance form was returned, 7,226 (47%) had a documented history of vaccination (6,312 with 1 dose and 914 with 2 doses), and 8,298 (53.4%) were unvaccinated

Table 1. Estimated proportion of mumps patients hospitalized and those given a code for complications by birth cohort, England and Wales, April 1, 2002–March 31, 2006*

Birth cohort	No. hospitalizations/total infections (%)	No. hospitalization complications/total infections (%)			
		Orchitis*	Meningitis	Pancreatitis	None
Pre-1980	451/4,455 (10.1)	158/2,376 (6.7)	26/4,455 (0.6)	21/4,455 (0.5)	260/4,455 (5.8)
1980–1989	1,782/35,152 (5.1)	811/18,814 (4.3)	118/35,152 (0.3)	114/35,152 (0.3)	791/35,152 (2.3)
1990–2006	414/3,737 (11.1)	27/1,496 (1.8)	10/3,737 (0.3)	11/3,737 (0.3)	367/3,737 (9.8)
Overall	2,647/43,344 (6.1)	996/22,686 (4.4)	154/43,344 (0.4)	146/43,344 (0.3)	1,418/43,344 (3.3)

*In male patients ≥12 years of age only.

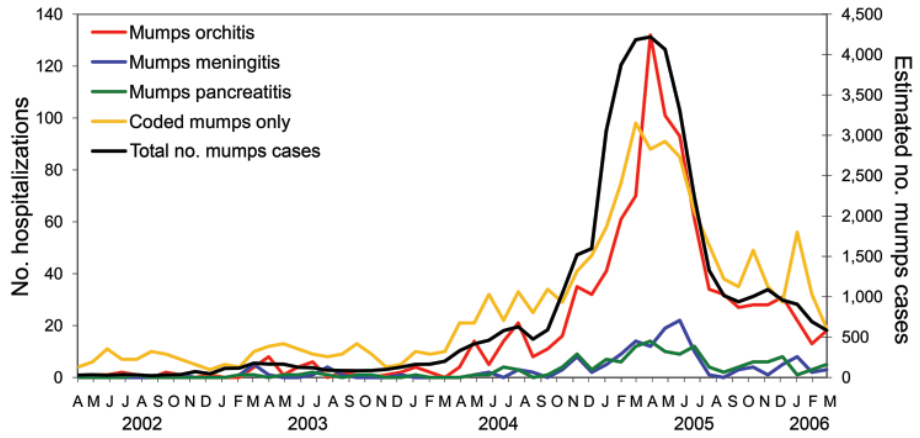

Figure. Total estimated number of cases of mumps and hospital episodes coded to mumps, England and Wales, April 1, 2002–March 31, 2006.

(many of whom were born before 1988 and therefore were not eligible for MMR). Hospitalization was noted for 452 (2.9%) mumps case-patients. The reported complication rate for mumps meningitis was 0.3% (53/15,524) and was 0.25% (38/15,524) for mumps pancreatitis. The most common complication was orchitis, reported for 6.1% (486/7,917) of male case-patients ≥12 years of age. The proportion of case-patients with each complication (excluding those with >1) that were hospitalized was lowest for mumps orchitis at 35.3% (166/470), followed by the proportion for mumps meningitis, 78.3% (36/46), and for mumps pancreatitis, 81.5% (22/27). The remaining 228 case-patients were hospitalized for varied reasons, ranging from airway concerns to anxious parents. The rate of hospitalization and rate of each main mumps complication were lower in those that were vaccinated than in the unvaccinated; rates were particularly low among those who had received 2 doses of vaccine (Table 2).

The ORs of reported hospitalization, orchitis, and meningitis were significantly lower in the vaccinated (1- or 2-dose MMR) than in the unvaccinated patients (Table 2). The polynomials for the age variable in the final logistic regression model for hospitalization, orchitis, meningitis. and pancreatitis are second, fourth, first, and second, respectively. Adjusting for age and sex had very little effect on the protective effect of vaccination in reducing the risk for hospitalization. The OR of having mumps meningitis was also found to be higher in male patients at 1.93 (95% CI 1.07–3.48) after vaccination status and age were controlled for.

Discussion

The mumps outbreak in England and Wales led to a clear increase in hospitalizations caused by mumps complications, which mirrored the outbreak curve. From April 1, 2002, through March 31, 2006, the estimated hospitalization rate from HES data was 6.1% overall. A much lower rate of hospitalization (2.9%) was derived from the enhanced surveillance forms. In contrast, the rate of mumps orchitis estimated from HES data was lower than that found by enhanced surveillance. This may be explained by the fact that most mumps orchitis cases were managed in primary care. Most reported case-patients with mumps meningitis and pancreatitis were admitted to the

Table 2. Association between receipt of vaccination and mumps complications, adjusted for age and sex, England and Wales, April 1, 2002–March 31, 2006*

Complication	Vaccine dose	No. cases/total cases (%)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Hospitalization	0	317/8,298 (3.8)	1	1
	1	122/6,312 (1.9)	0.50 (0.40–0.61)	0.54 (0.43–0.68)
	2	13/914 (1.4)	0.36 (0.21–0.64)	0.45 (0.25–0.80)
Orchitis†	0	356/4,574 (7.8)	1	1
	1	123/3,241 (3.8)	0.44 (0.36– 0.55)	0.72 (0.56–0.93)
	2	7/475 (1.5)	0.17 (0.08–0.37)	0.64 (0.28–1.44)
Meningitis	0	42/8,298 (0.5)	1	1
	1	10/6,312 (0.2)	0.31 (0.16–0.62)	0.28 (0.14–0.56)
	2	1/914 (0.1)	0.22 (0.03–1.57)	0.17 (0.02–1.26)
Pancreatitis	0	26/8,298 (0.3)	1	1
	1	12/6,312 (0.2)	0.61 (0.31–1.20)	0.95 (0.41–2.19)
	2	0/914	0 (0–1.34)‡	–

*OR, odds ratio; CI, confidence interval; –, not estimable.
†Adjusted for age only.
‡Exact CI.

hospital, but the estimated rate of these complications was low (<0.5%) by using either method.

On the basis of the rate of hospital episodes and data from enhanced surveillance, the complication rates observed here are low in comparison to results of studies from the prevaccine era. Previously published complication rates for mumps suggest that orchitis is the most common complication in 15%–30% of adult men with mumps (*21*–*24*). Mumps meningitis has been reported in 1%–10%, mumps pancreatitis in 4%, and mumps oophoritis in 5% of persons with mumps (*3*,*25*,*26*). The much lower rates observed in our study likely reflect the fact that the denominator is derived from population-based surveillance which aims to capture all cases of diagnosed mumps. Because the United Kingdom provides free universal access to primary care, we were able to ascertain milder cases that may not have been included in studies that use secondary care data or in studies conducted in other countries.

The estimated complication rates were lower in younger persons, particularly in the cohorts eligible for mumps vaccination. The outbreak in England and Wales during 2004–2005 affected mainly those born from 1980 through 1989 (*1*,*8*). Only those born in the second half of the 1980s could have been offered MMR; either routinely in the second year of life (those born from 1987 onwards) or as a catch-up at school entry for those who had not received measles vaccine. Those born after 1989 were eligible for routine MMR at 13 months and for a second dose of MMR at school age when it was introduced in 1996. The lower estimated hospitalization rate for mumps orchititis in younger cohorts could be attributed to less severe disease in younger persons or to the effect of mumps vaccination. The latter explanation was supported by the finding that a history of mumps vaccination was also associated with a lower risk for mumps hospitalization, mumps orchitis, and mumps meningitis in the enhanced surveillance data, even after age and sex were controlled for. Our analysis suggests that the adjusted odds of being hospitalized with mumps are reduced ≈50% in those with a history of at least 1 mumps vaccination. We observed an even lower rate of hospitalization in those who had received 2 doses than in those who had received 1 dose of vaccine, although this difference was not significant. Male patients had a higher risk for mumps meningitis, even after vaccination status was adjusted for. Results of vaccine effectiveness studies and the long-term persistence of mumps antibody have not shown differences on the basis of sex (*7*,*12*,*26*). However, mumps meningitis has been shown to affect male patients more often than female patients (*25*).

Most published complication rates derive from the prevaccine era; however, almost half of the case-patients included in our enhanced surveillance had been vaccinated. Our findings are more consistent with those of other studies in the MMR era in which rates of orchitis in postpubertal male patients were 10%–12%, and the rate of mumps meningitis was 0.9% (*11*,*12*). To our knowledge, information on the association between mumps vaccination and mumps complications is limited. A study of outbreaks of mumps in US colleges in 2006 showed no significant association between vaccination status and complications in a highly vaccinated population (*11*). The larger sample size in our analysis allowed us to detect differences in complication rates by vaccination status, which may be undetectable in smaller studies or when the number of unvaccinated persons is low. A limitation of the enhanced surveillance database is the possible bias from nonresponses. The higher response rates in younger, vaccinated persons would be expected to improve ascertainment of complications in this cohort. However, we observed lower complication rates in the young and vaccinated, which suggests that our observations are not due to response bias.

We believe it is plausible that vaccination against mumps can lead to a shift toward milder forms of the disease in a similar way as has been observed with varicella vaccine (*27*). Natural mumps in unvaccinated persons is known to be manifested as a minimally symptomatic infection with viral shedding (*28*). Studies have also reported a high proportion of asymptomatic or minimally symptomatic infections among vaccinated persons; more than half of case-patients did not have classical parotitis (*12*). The possibility of reduced severity of infections in vaccinated case-patients is also supported by findings of a lower virus isolation rate and shorter duration of viral detection in studies that compare vaccinated to unvaccinated patients (*29*,*30*). The lower rates of complications in vaccinated teenagers and young adults are consistent with secondary vaccine failure, which suggests that the primed person is able to mount an immune response to prevent more serious complications. A large number of cases with secondary vaccine failure is also consistent with declining protection with time since vaccination (*7*,*31*).

By using HES data, however, we could have underestimated the rates of complications because a substantial number of hospitalizations were coded for mumps alone. The overall rate based on hospital episodes is probably a high estimate because the numerator derives from an exhaustive database, whereas the denominator was derived from number of confirmed cases, a category that is prone to some underreporting. To minimize this underreporting effect, we combined clinical notifications during a period of high positive predictive value with laboratory-confirmed mumps cases derived from population-based surveillance by using noninvasive oral fluid testing. The use of laboratory-confirmed mumps cases based on serologic testing alone in the denominator is likely to overrepresent hospitalized case-patients and therefore to

overestimate complication rates. The rate from enhanced surveillance is more likely to be a true reflection of absolute rate because both numerator and denominator are derived from the same source. In addition, although both estimates are dependent on patients seeking care for the complication, clinical details in the enhanced surveillance were supplied directly from primary care physicians who had diagnosed mumps. Therefore, complications exhibited some time after infection were less likely to be attributed to mumps.

The effects of long-term complications, such as sensorineural deafness and the possible link between mumps orchitis and infertility, were not included in our analysis (*3*,*32*–*35*). With the current outbreaks in colleges as well as in other congregate settings, mumps orchitis in postpubertal young men may require further research. A concern exists that mumps epididymitis (which carries a risk for testicular damage with subsequent infertility) is easily misdiagnosed as orchitis (*36*). As reports of mumps outbreaks in highly immunized populations of older teenagers and young adults continue to occur, the long-term effects of mumps complications may be substantial. Our analysis, however, suggests that vaccination provides higher levels of protection against hospitalization and risk for orchitis and meningitis in those diagnosed with mumps. The effect of vaccination on mumps complications will therefore be increasingly critical in assessing the outcome of current and future mumps control strategies.

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Molecular Epidemiology of *Coxiella burnetii* from Ruminants in Q Fever Outbreak, the Netherlands

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Q fever is a zoonosis caused by the bacterium *Coxiella burnetii*. One of the largest reported outbreaks of Q fever in humans occurred in the Netherlands starting in 2007; epidemiologic investigations identified small ruminants as the source. To determine the genetic background of *C. burnetii* in domestic ruminants responsible for the human Q fever outbreak, we genotyped 126 *C. burnetii*–positive samples from ruminants by using a 10-loci multilocus variable-number tandem-repeat analyses panel and compared them with internationally known genotypes. One unique genotype predominated in dairy goat herds and 1 sheep herd in the human Q fever outbreak area in the south of the Netherlands. On the basis of 4 loci, this genotype is similar to a human genotype from the Netherlands. This finding strengthens the probability that this genotype of *C. burnetii* is responsible for the human Q fever epidemic in the Netherlands.

Q fever is a zoonosis caused by *Coxiella burnetii*, an intracellular gram-negative bacterium that is prevalent throughout the world (*1*). Domestic ruminants are considered the main reservoir for Q fever in humans (*2*). However, other animal species, including pet animals, birds, and several species of arthropods, can be infected by *C. burnetii* and cause human cases of Q fever (*2–5*). The main clinical manifestations of Q fever in goats and sheep are abortion and stillbirth. In cattle, Q fever has been associated with sporadic abortion, subfertility, and metritis (*4*,*6*). With an abortion, up to 1 billion *C. burnetii* per gram of placenta can be excreted (*7*). Most animal species that carry *C. burnetii* show no symptoms (*4*). Transmission to humans occurs mainly through inhalation of contaminated aerosols (*4*,*5*,*8–10*).

Recently, 2 DNA-based methods for typing *C. burnetii* were reported (*11–13*). Multispacer sequence typing is based on DNA sequence variations in 10 short intergenic regions and can be performed on isolated *C. burnetii* strains or directly on extracted DNA from clinical samples (*12*,*14*,*15*). Multilocus variable-number tandem-repeat analyses (MLVA) is based on variation in repeat number in tandemly repeated DNA elements on multiple loci in the genome of *C. burnetii* and might be more discriminatory than multispacer sequence typing (*13*,*15*). MLVA also can be performed on *C. burnetii* strains (*11*,*15*) or directly on DNA extracted from clinical samples (*16*). A total of 17 different minisatellite and microsatellite repeat markers have been described (*11*).

Starting in 2007, the Netherlands has been confronted with one of the largest Q fever outbreaks in the world, involving 3,921 human cases in 4 successive years. On 28 dairy goat farms and 2 dairy sheep farms, abortion storms (with abortion rates up to 80%) caused by Q fever were diagnosed during 2005–2009. These small ruminants are considered the source of the human Q fever outbreak in the Netherlands (*17*). The connection between Q fever abortion storms in small ruminants and human Q fever cases is based primarily on epidemiologic investigations (*18–21*). A limited investigation by genotyping with MLVA recently showed that farms and humans in the

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Netherlands are infected by multiple different, yet closely related, genotypes of *C. burnetii* (*16*).

Although dairy goats and dairy sheep appear to be the source of the human Q fever outbreak in the Netherlands, no information is available about the genetic background of *C. burnetii* in these populations. This knowledge is essential for gaining insight into the molecular epidemiology of the organism and the origin of the outbreak, as well as for outbreak management purposes.

Our objective was to show the genetic background of *C. burnetii* in domestic ruminants responsible for the human Q fever outbreak. This information is necessary to evaluate the epidemiologic link between the source and human cases and to compare the outbreak genotypes with internationally known genotypes. During 2008–2010, a total of 125 *C. burnetii*–positive samples from 14 dairy goat farms, 1 dairy cattle farm, and 2 sheep farms were typed by MLVA. In addition, we show the geographic distribution of these *C. burnetii* genotypes across the Netherlands and compare the genotypes with what is internationally known.

Materials and Methods

Animal Samples

Our study comprised 14 dairy goat farms (farms A–E, H, J, M, N, O, P, Q, AE, and AF), 1 dairy cattle farm (farm R), and 2 sheep farms (1 dairy sheep farm Y and 1 sheep farm Z) sampled during the Q fever outbreak in the Netherlands (Table 1; Figure 1). On 12 of the 14 dairy goat farms, multiple abortions had occurred. On 2 dairy goat farms (farms J and M) and on the dairy sheep farm (farm Y), no abortions had occurred. On 1 dairy cattle farm and on the sheep farm (farm Z), *C. burnetii* was detected in a placenta after abortion. One goat farm (farm AG) sampled in 2001 was included with an archived histologic section

Table 1. Overview of *Coxiella burnetii* genotyping results for farms sampled during human Q fever outbreak, the Netherlands, 2007–2010*

Farm ID	Animal species	Approximate herd size	Year of sampling	Approximate abortions in year of sampling, %	Sample type	No. samples tested	No. samples included in study	MLVA typing results	
								MLVA ID	No. samples
A	Dairy goats	617	2008	25	Vaginal swabs	20	9	CbNL01	7
								CbNL05	1
								CbNL07	1
B	Dairy goats	598	2008	20	Vaginal swabs	20	5	CbNL01	5
C	Dairy goats	546	2008	25	Vaginal swabs	20	20	CbNL01	20
D	Dairy goats	1,498	2008	19	Vaginal swabs	39	7	CbNL01	6
								CbNL04	1
E	Dairy goats	1,568	2008	8 (2007)	Fetal tissue	3	3	CbNL01	1
								CbNL09	1
								CbNL11	1
H	Dairy goats	606	2008	80	Vaginal swabs	13	8	CbNL01	7
								CbNL02	1
J	Dairy goats	459	2008	None	Vaginal swabs	3	3	CbNL01	2
								CbNL08	1
M	Dairy goats	769	2008	None	Vaginal swabs	2	1	CbNL10	1
N	Dairy goats	1,187	2009	25	Vaginal swabs	20	20	CbNL01	20
					Placenta	1	1	CbNL01	1
O	Dairy goats	83	2009	7	Vaginal swabs	40	16	CbNL01	14
								CbNL03	1
								CbNL06	1
					Milk	1	1	CbNL01	1
P	Dairy goats	548	2009	10	Vaginal swabs	20	6	CbNL01	6
Q	Dairy goats	340	2009	10	Vaginal swabs	25	19	CbNL01	19
AE	Dairy goats	500	2007	>5	Placenta	1	1	CbNL12	1
AF	Dairy goats	2,000	2007	>5	Placenta	1	1	CbNL01	1
AG	Dairy goats	590	2001	>5	Paraffin-embedded placenta	1	1		1
R	Dairy cattle	70	2007	<5	Placenta	1	1	CbNL13	1
Y	Dairy sheep	184	2010	None	Vaginal swabs	5	1	CbNL10	1
					Bulk tank milk sample	1	1	CbNL10	1
Z	Sheep	2	2009	50	Placenta	1	1	CbNL01	1

*ID, identification; MLVA, multilocus variable-number tandem-repeat analysis.

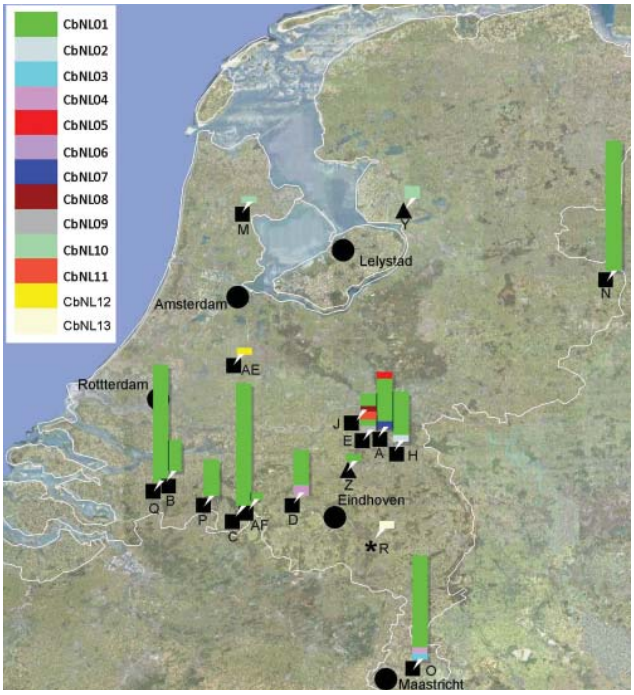

Figure 1. Map of the Netherlands showing locations of farms sampled during the Q fever outbreak, 2007–2010. Farms are indicated by letter and ruminant species (black squares, goats; black triangles, sheep; black star, cattle); genotypes of *Coxiella burnetii* found per farm are indicated by bars at each farm's location. The height of the bar indicates numbers of isolates per genotype.

of paraffin-embedded placenta from an abortion outbreak caused by *C. burnetii* infection.

Vaginal swabs and milk samples from dairy goats and dairy sheep were sent to the national reference laboratory for notifiable animal diseases (the Central Veterinary Institute, part of Wageningen UR) by the Dutch Food and Consumer Product Safety Authority in accordance with the regulation in place at that time. These samples were submitted for confirmation testing of farms with clinically suspected Q fever (farms A–D, N, O, P, and Q), for tracing the source of human Q fever cases (because of proximity to human case-patients, farms H, J, and M) or for bulk tank milk monitoring (farm Y). Samples of immunohistochemically confirmed Q fever–positive goat and sheep placentas (farms N, AE, AF, and Z) and fetal tissue (farm E) were provided by the Animal Health Service, including 1 archived histologic section of paraffin-embedded placenta from a *C. burnetii* abortion outbreak in a goat farm in 2001 (farm AG), which was diagnosed retrospectively (*22*). The sampled dairy goat farms represent 60% of the farms with known abortion problems during 2007–2009.

Testing of Samples before MLVA Typing

DNA was extracted from vaginal swabs and milk by using Chelex resin (InstaGene; Bio-Rad, Hercules, CA, USA). A vaginal swab tip or 200 μL of milk was added to 400 μL of Chelex suspension and incubated and shaken for 30 min at 56°C, followed by an incubation step for 8 min at 100°C. The clarified supernatant was used for PCR and MLVA. DNA from placentas was extracted by using a DNA tissue kit (DNeasy Blood and Tissue Kit; QIAGEN, Hilden, Germany). DNA from the paraffin-embedded placenta was extracted by using MagneSil Genomic Fixed Tissue System (Promega, Madison, WI, USA).

All samples were tested by an in-house real-time PCR directed toward the *C. burnetii*–specific IS*1111a* element (*23*). An inhibition control was constructed by using the primes of the IS*1111a* element (Table 2). PCR was performed on a 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA) by using 400 nmol/L of primers and 200 nmol/L of probes in 7 μL PerfeCTa Multiplex qPCR Supermix, uracil-N-glycosylase (2×) (New England Biolabs, Ipswich, MA, USA] with Low Rox dye (Quanta BioSciences, Gaithersburg, MD, USA), 1 μL of inhibition control, 5 μL of sample, and 7 μL of water. An initial uracil DNA glycosylase (UDG) incubation for 5 min at 45°C and denaturation/activation for 60 s at 95°C was followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 60°C. Results were generated with 7500 Fast System Software (Applied Biosystems).

MLVA Typing

MLVA typing was performed by using a selection of 10 of the 17 loci described by Arricau-Bouvery et al. (*11*) according to the Multiple Loci VNTR Analysis databases for genotyping (http://minisatellites.u-psud.fr/MLVAnet/querypub1.php), except that Ms12 was omitted because of poor performance, and Ms24 was added (Table 2). New primers were designed for Ms27 and Ms28 to improve performance. The annotation of Ms30, Ms31, and Ms36 was updated (P. Le Flèche, pers. comm.).

The PCR amplification was performed by using an Applied Biosystems 9700 thermocycler in a total volume of 25 μL containing 1× reaction buffer, 1 U True Start *Taq* DNA polymerase (Fermentas, Glen Burnie, MD, USA), 2 mmol/L $MgCl_2$, 0.2 mmol/L of each nucleotide (dATP, dGTP, dCTP, dUTP), 0.5 μmol/L of each primer, 0.5 U UDG (New England Biolabs), and 2–5 μL template. An initial UDG incubation for 5 min at 37°C and denaturation/activation for 2 min at 95°C was followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60/65°C, elongation for 30 s at 72°C, followed by a final extension step for 5 min at 72°C. After the amplification, 0.5 U UDG inhibitor (New England Biolabs) was added to the PCRs to prevent further UDG activity. Up to 4 different PCR products with different fluorescent dyes were diluted, depending on the PCR efficiency, and pooled. From these pooled PCR products, 4 μL was mixed with 15 μL

Table 2. Primers and probes used in the PCR for detecting *Coxiella burnetii* in clinical samples and loci and primers for MLVA of *C. burnetii*, the Netherlands, 2007–2010*

Identification	Temp, °C	Primer sequence for MLVA, with label indicated, 5′ → 3′	
		Forward	Reverse
Primers IS*1111a*	60	CATCACATTGCCGCGTTTAC	GGTTGGTCCCTCGACAACAT
Probe IS*1111a*	60	AATCCCCAACAACACCTCCTTATTCCCAC	
Probe inhibition control	60	ACATAATCTCTCCGACCCCACACTTCCATAC	
Cbu0448_ms03_12bp_7U_229bp	60	6-FAM-TTGTCGATAAATCGGGAAACTT	CACTGGGAAAAGGAGAAAAAGA
Cbu1963_ms21_12bp_6U_210bp	60	NED-AGCATCTGCCTTCTCAAGTTTC	TGGGAGGTAGAAGAAAAGATGG
Cbu1980_ms22_11bp_6U_246bp	60	PET-GGGGTTTGAACATAGCAATACC	CAATATCTCTTTCTCCCGCATT
Cbu0259_ms24_7bp_27U_344bp	65	VIC-ATGAAGAAAGGATGGAGGGACT	GATAGCCTGGACAGAGGACAGT
Cbu0838_ms27_6bp_4U_320bp†	65	6-FAM-GGGTCAGGTGGCGGGTGTG	TTCTCGCAAACGTCGCACTAACTC
Cbu0839_ms28_6bp_6U_480bp†	60	VIC-TAGAAACCGATAATCCCCTTGACA	ATTCCGCCGCCATTGAG
Cbu1351_ms30_18bp_6U_306bp‡	60	NED-ATTTCCTCGACATCAACGTCTT	AGTCGATTTGGAAACGGATAAA
Cbu1418_ms31_7bp_5U_285bp‡	60	PET-GGGCATCTAATCGAGATAATGG	TTTGAGAAAATTTTGGGTGCTT
Cbu1471_ms34_6bp_5U_210bp	60	6-FAM-TGACTATCAGCGACTCGAAGAA	TCGTGCGTTAGTGTGCTTATCT
Cbu1941_ms36_9bp_4U_477bp‡	65	VIC-GAAACCAGTCTTCCCTCAACAG	ATAACCGTCATCGTCACCTTCT

*MLVA, multilocus variable-number tandem-repeat analyses; temp, annealing temperature.
†Different primer set from the proposed set by Arricau-Bouvery et al. (*11*).
‡Updated after personal communication with Le Flèche, Université Paris-Sud, Orsay Cedex, France.

of Hi-Di formamide (Applied Biosystems) and 0.5 μL of GeneScan 600 LIZ Size Standard (Applied Biosystems). After denaturation for 3 min at 96°C the samples were cooled on ice. The PCR products were separated on a 3130 Genetic Analyzer (Applied Biosystems) with a 36-cm array by using POP7 polymer.

The fragments were sized by using GeneMapper version 4.0 software (Applied Biosystems). The accuracy of the sizing obtained by capillary electrophoresis was determined by comparing sequencing data from the reference strain with the obtained fragment size from the capillary electrophoresis and corrected if necessary. The number of repeats for each locus was determined on the basis of the published and corrected annotation of the various loci (Table 2). Non–whole repeat numbers were rounded off mathematically. Reproducibility was checked with positive controls.

Data Analysis

The reference strain Nine Mile was used as reference (*11*). Analyses were performed, including only genotypes of *C. burnetii* containing ≤2 loci with missing values. Numerical typing data were imported into BioNumerics v 6.1 (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed with the multistate categorical similarity coefficient by using unweighted pair group method with arithmetic mean clustering. Missing values were imported as question marks. The genotypic diversity of the population under study was calculated by using the adapted Simpson index of diversity (Hunter-Gaston diversity index [HGDI]) (*11*,*24*).

Found MLVA patterns based on the number of repeats per locus were called MLVA types and identified as CbNLxx. We compared MLVA types with MLVA types in the publicly accessible Multiple Loci VNTR Analysis databases for genotyping: Coxiella2007 and Coxiella2009_Netherlands (access date 2001 Jan 11). The Nine Mile strain was used as reference.

Results

The study comprised 122 samples from 15 dairy goat farms, 2 samples from 1 dairy sheep farm, and 1 sample each from 1 sheep farm and 1 dairy cattle farm were included in this study (Table 1). Of the farms sampled during the outbreak, 13 were situated in the southern part of the Netherlands; 3 dairy goat farms (farms M, N, and AE) and 1 dairy sheep farm (farm Y) were located outside this area (Figure 1). From the 238 Q fever PCR-positive samples from the farms in this study, 125 (53%) yielded a genotype with ≤2 missing values: 52 with a complete genotype, 48 with 1 missing value, and 25 with 2 missing values. 113 (47%) PCR-positive samples represented partial genotypes with 3–10 missing values. From the paraffin-embedded placenta (farm AG), only a partial genotype could be shown, with 6 repeats on Ms03 and 10 repeats on Ms34.

We distinguished 13 genotypes in the 125 samples (CbNL01–CbNL13; Table 1; Figures 1, 2). All *C. burnetii* genotypes could be associated with abortion, except for 2 (CbNL10, farm M and Y; and CbNL08, farm J; Figure 1). The relationship between the genotypes in all samples is shown in Figure 2, including the genotype of the reference strain Nine Mile and the reference genotype of the reference strain Nine Mile from Arricau-Bouvery et al. (*11*), which were identical.

The 13 genotypes are separated in 2 clusters (Figure 2). One cluster containing a genotype represented by 111 (90%) of the samples (CbNL01); 1 genotype (CbNL10) represented by 3 samples (1 from a dairy goat farm and 2 from a dairy sheep farm); and 10 genotypes (CbNL02–CbNL09 and CbNL11) represented by 1 sample, all

from dairy goat farms. The second cluster was distinctly separated from the other cluster, representing 2 genotypes in 1 dairy goat sample (CbNL12), in 1 dairy cattle sample (CbNL13) and the paraffin-embedded placenta. In samples from dairy goat farms with abortion problems, the same genotype (CbNL01) was present in 110 (91%) of 121 samples. One sheep sample also showed this genotype (farm Z). The geographic distribution of the genotypes according to the location of the originating farm is given in Figure 1. The relationship between the genotypes found in this study and the internationally known genotypes are presented in the phylogenetic trees in Figure 3 on the basis of 4 loci and in Figure 4 on the basis of 9 loci.

Discussion

We performed MLVA typing of *C. burnetii* based on 10 loci on a large number of Q fever–positive samples to show the genetic background of *C. burnetii* in the domestic ruminants associated with the Q fever outbreak in humans in the Netherlands. In 125 (53%) of 237 samples, an adequate genotype for *C. burnetii* was generated. Previously, MLVA typing was performed on *C. burnetii* strains after primary isolation and cultivation (*11*,*13*,*15*) or, in the Netherlands, on only 11 clinical samples from humans, sheep, and goats with a selected number of 3 loci (*16*).

The main drawback of typing on clinical samples is the variable quality and amount of DNA. These drawbacks influence the typability of samples, resulting in partial genotypes; whether the missing values are caused by insufficient DNA concentrations and quality or by an absence of loci is unclear. If loci are absent, partial genotypes also are expected to be found in samples with high DNA loads. Such is not the case in our study. Typing of placenta material that contains high quantities of *C. burnetii*, as well as vaginal swabs with PCR cycle threshold (C_t) values <32, yielded complete genotypes. In samples with C_t values of 32–34, only partial genotypes were obtained. Samples with a C_t value >34 were poorly typable.

Arricau-Bouvery et al. (*11*) calculated diversity indices for the 17 loci used in the MLVA, which varied from 0.28 for locus Ms22 to 0.86 for locus Ms34. The HGDI for the combined panels 1 and 2 of the MLVA typing method for *C. burnetii* can be calculated on 0.99 and for panel 2 on 0.92. These HGDIs are in the upper part of the 0.438–0.997 range reported by Hunter and Gaston (*24*) for typing methods for various bacteria and yeasts.

The high diversity indices for the MLVA of *C. burnetii* indicate a high discriminating power, and this capability makes MLVA typing suitable for distinguishing *C. burnetii* isolates. With this highly discriminatory typing method, we found that 1 genotype of *C. burnetii* predominated on all dairy goat farms in the southern part of the Netherlands. On 12 of 14 dairy goat farms, this genotype was found in 91% of samples, varying per farm from 33% (farm E) to 100% (farms B, C, N–Q, Table 1, Figures 1 and 2). Although the sample size was small compared with the number of animals on the farm (Table 1), these data show that 1 genotype was far more common than other genotypes found on these farms. The 9 other genotypes occurred once, each representing only 0.8% of all found genotypes on dairy goat farms. The most predominant genotype was found on all 11 dairy goat farms in the southern Netherlands and on a farm in the eastern part of the country (farm N). This finding strongly suggests a clonal spread of *C. burnetii* with this predominant genotype over the dairy goat farms in the southeastern part of the Netherlands.

The clonal spread of 1 genotype of *C. burnetii* could be explained by 2 phenomena. First, the dairy goat industry in the Netherlands sharply increased from almost 100,000 dairy goats in 2000 to >230,000 dairy goats on

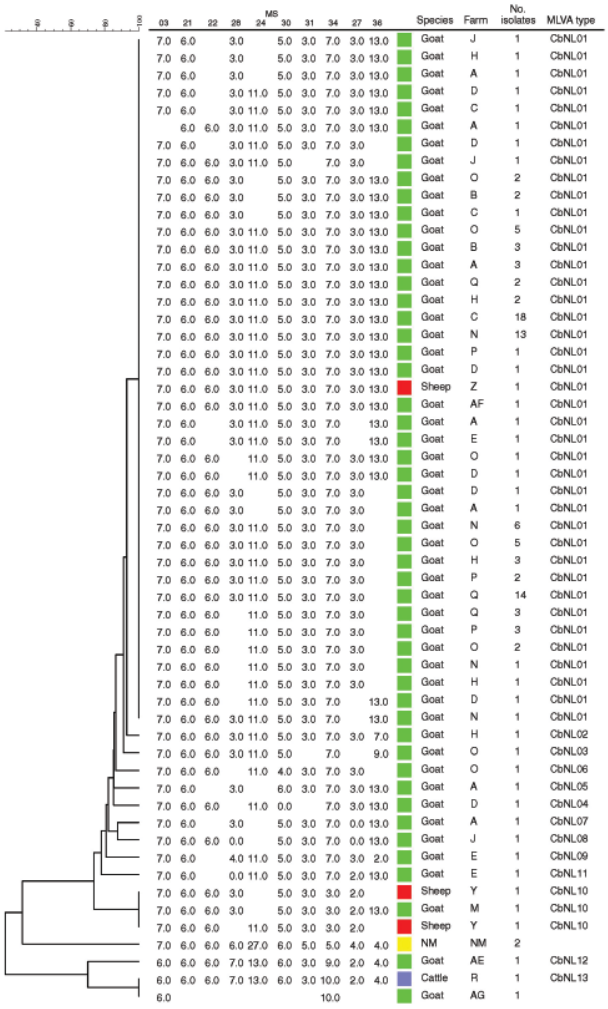

Figure 2. Phylogenetic tree with genotypes of *Coxiella burnetii* of all samples in the study, the Netherlands, on the basis of 10 multilocus variable-number tandem-repeat analyses (MLVA). Repeats per locus are shown; open spots indicate missing values. NM, Nine Mile reference strain.

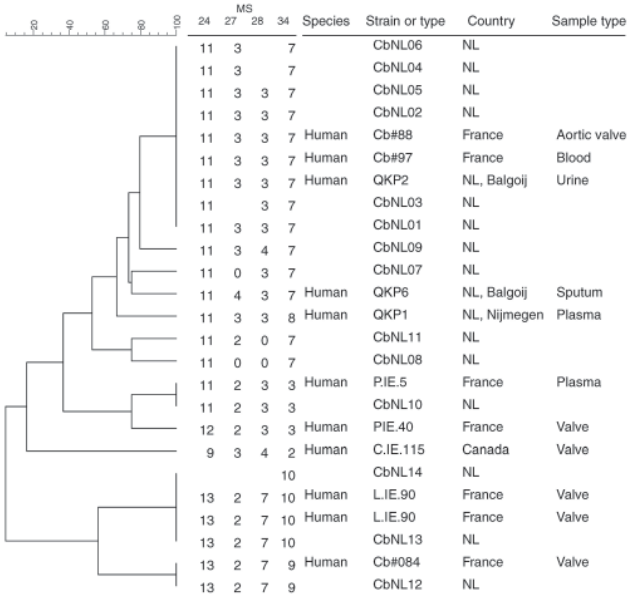

Figure 3. Phylogenetic tree with genotypes of *Coxiella burnetii* that are most closely related to the Dutch genotypes on the basis of 4 multilocus variable-number tandem-repeat analyses (MLVA). Genotypes are derived from the Multiple Loci VNTR Analysis databases for genotyping (http://minisatellites.u-psud.fr/MLVAnet/querypub1.php: Coxiella2009_Netherlands [accessed 2011 Jan 11]). Repeats per locus are shown; open spots indicate missing values. NL, the Netherlands.

≈350 farms in 2009 (*17*). Most of these goats were bred in the Netherlands, which probably resulted in a microbial relationship between many of the dairy goat herds. In this theory, the *C. burnetii* strain with the most predominant genotype was present in the Netherlands for a long period before the abortion problems in dairy goats started in 2005. This theory is not supported by the results of the typing of the paraffin-embedded placenta from an aborted dairy goat who in 2001. The typing result differs on 2 loci from the most predominant genotype found in this study. Second, clonal spread could have been facilitated by emergence of a genotype of *C. burnetii* causing abortion in dairy goats that could then spread successfully over the dense goat population in the southeastern part of the country. Whether this genotype is more virulent is subject to research.

On the basis of comparison of MLVA types on 4 loci (Figure 3), CbNL01–06 could not be distinguished and were similar to the genotype of a person in the Netherlands (QPK2) and 2 genotypes from persons in France (Cb#88, Cb#97). The sample from a person in the Netherlands is derived from patient 2 reported by Klaassen et al. (*16*). Patient 2 is the farmer of farm A, where genotype CbNL01 predominated, as well as CbNL05 (Table 1). This shows a genetic link between the *C. burnetii* DNA from the farmer and his abortive goats, which suggests that the farmer was infected by his own goats. However, this link is based on only 4 loci on 1 human sample. To further confirm the link between dairy goats and humans, more samples need to be typed with more MLVA loci to increase the discriminatory power.

The human sample with ID QKP6 is the same sample as that from patient 4 reported by Klaassen et al. (*16*) and is most closely related to CbNL07. Human sample QKP1 is the same as that of patient 1. Patient 5 fits in the genotype cluster in the Netherlands, as does patient 2. The sheep reported by Klaassen et al. did not abort, and their samples show a difference of 1 repeat on Ms34 compared with CbNL01. On the basis of the comparison of MLVA types on 9 loci (Figure 4), all genotypes in this study can be distinguished. The most predominant genotype CbNL01 clusters with other genotypes (CbNL02–CbNL09, CbNL11) and with 1 human sample (Cb#97) from France. CbNL01 differed from this human isolate on 2 loci

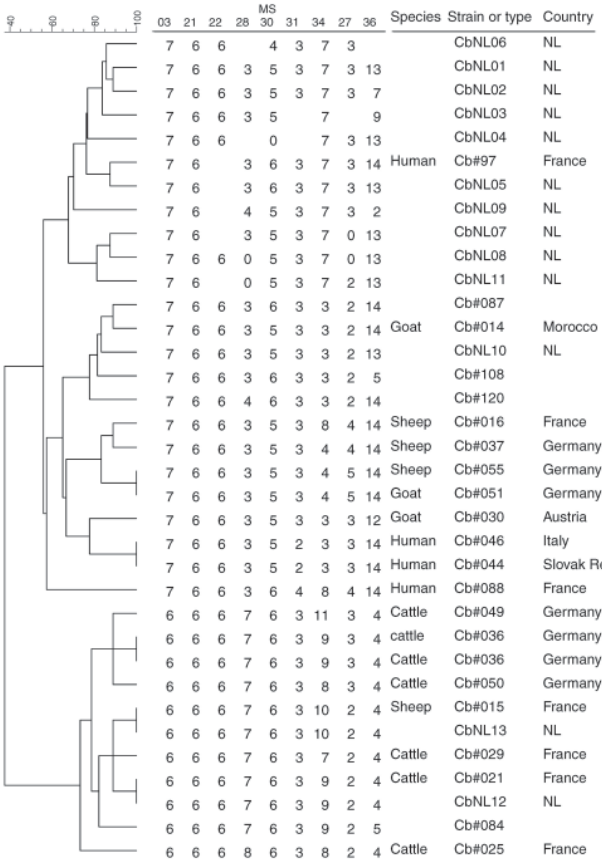

Figure 4. Phylogenetic tree with genotypes of *Coxiella burnetii* that are most closely related to the Dutch genotypes on the basis of 9 multilocus variable-number tandem-repeat analyses (MLVA). Genotypes are derived from the Multiple Loci VNTR Analysis databases for genotyping (http://minisatellites.u-psud.fr/MLVAnet/querypub1.php: Coxiella2007 [accessed 2011 Jan 11]). Repeats per locus are shown; open spots indicate missing values. NL, the Netherlands; Slovak Rep, Slovak Republic.

(Ms30 and 36), which shows that the most predominant genotype in the Netherlands is unique. Whether this finding can be attributed to the small number of strains and clinical samples typed or is really a unique genotype is not yet clear. The closest relation to an isolate from France might give a clue about the origin of the genotype from the Netherlands.

The human Q fever outbreak in the Netherlands started in the southern part of the country and resulted in >3,500 human cases during 2007–2010. Dairy goats and dairy sheep are considered to be the source of this outbreak, primarily on the basis of epidemiologic findings (*10*,*17*,*20*,*21*,*25*,*26*). In our study, samples were typed from farms suspected of being the source of the human Q fever outbreak. Results show that 1 genotype of *C. burnetii* predominated in the dairy goats and sheep in the human Q fever outbreak area in the southern part of the Netherlands, and this genotype also was present in a human case-patient in the Netherlands. This *C. burnetii* genotype is expected to have played a key role in the Q fever outbreak in small ruminants in the Netherlands and was also transmitted widely to humans, causing Q fever in the human population. If this hypothesis holds true, *C. burnetii* with the same genotype as in dairy goats should be found in most samples from human Q fever patients. To this end, a study was performed to show the genetic background of human *C. burnetii* isolates in the Netherlands by using a concordant MLVA typing method (J.J.H.C. Tilburg et al., unpub. data). Furthermore, the uniqueness of the predominant genotype of *C. burnetii* for the Netherlands can be part of the explanation why the magnitude of the Q fever outbreak in the Netherlands has never been seen elsewhere.

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Remaining Questions about Clinical Variola Major

J. Michael Lane

After the recent summary of World Health Organization–authorized research on smallpox, several clinical issues remain. This policy review addresses whether early hemorrhagic smallpox is disseminated intravascular coagulation and speculates about the cause of the high mortality rate among pregnant women and whether ocular smallpox is partly the result of trachoma or vitamin A deficiency. The joint destruction common in children with smallpox might be prevented by antiviral drugs, but intraarticular infusion of antiviral drugs is unprecedented. Development of highly effective antiviral drugs against smallpox raises the issue of whether postexposure vaccination can be performed without interference by an antiviral drug. Clinicians should consider whether patients with smallpox should be admitted to general hospitals. Although an adequate supply of second-generation smallpox vaccine exists in the United States, its use is unclear. Finally, political and ethical forces suggest that destruction of the remaining stocks of live smallpox virus is now appropriate.

After the World Health Organization (WHO) declared smallpox eradicated in 1980, several problems remained concerning the disease and its causative virus, variola major virus. These problems included high rates of adverse events associated with most strains of vaccinia virus; our inadequate understanding of the pathophysiology of smallpox; lack of a good animal model of the disease; difficulty of rapid laboratory diagnosis, including the poor ability of most standard laboratory tests to distinguish between orthopoxviruses; lack of an effective antiviral drug; and rudimentary knowledge about the genetic makeup of the virus.

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Although many observers wished to destroy the remaining stocks of variola major virus in 1980, several respected researchers wanted to use the live virus to help answer some of these remaining questions. In 1999, WHO agreed to a research agenda, with oversight by a WHO committee, to continue research with live variola major virus until substantial progress was made on these questions. The WHO oversight committee has now declared that satisfactory (if in some areas imperfect) progress has been made toward developing improved vaccines, better laboratory diagnostics, a reasonable nonhuman primate animal model, effective antiviral drugs, and good understanding of the genetics of orthopoxviruses. Reports of this progress have been published (*1*–*5*).

Despite this impressive progress, clinicians have been left with several unanswered questions. However, these questions may never be answered because we hope that there will never be another patient with classical smallpox, and the best nonhuman primate model does not perfectly reproduce clinical smallpox.

Is Early Hemorrhagic Smallpox Disseminated Intravascular Coagulation?

Smallpox was eradicated before cases of early hemorrhagic disease could be studied in modern clinical settings. This disease had all the hallmarks of disseminated intravascular coagulation (DIC). Patients had widespread hemorrhaging in the skin during the early septic phase, usually before a rash developed. Bleeding occurred from multiple orifices. Although necropsy evidence is minimal, internal bleeding likely affected many organs. Results of studies of bleeding time, clotting time, and tourniquet tests in patients with early hemorrhagic smallpox were consistent with what might be expected with DIC (*6*). In addition, patients experienced high fever, cardiovascular

collapse, and other clinical signs that we now associate with the cytokine cascade. Treating DIC remains difficult, but the case-fatality rate of early hemorrhagic smallpox approached 100%, and it might be reduced if treatment for DIC is instituted.

Are High Case-Fatality Rates in Unvaccinated Pregnant Women and Fetal Wastage a Result of Immune Suppression?

Evidence has shown that the death rate from smallpox among pregnant women was extraordinarily high. Pregnant women had a higher rate of hemorrhagic disease than did other adults. Approximately 16% of cases in unvaccinated pregnant women were early hemorrhagic smallpox versus ≈1% in nonpregnant women and adult males. The case-fatality rate in unvaccinated pregnant women approached 70%. Fetal wastage approached 80% (*6*–*9*).

We now know that a normal pregnancy includes a modest transient immune deficit, particularly suppression of Th1 and Tc cells (*9*). If therapeutic interventions were available that could assist the immune system during infection with smallpox virus, the case-fatality rate in pregnant women might be reduced considerably.

Are Ocular Variola and Its Resulting Blindness a Result of Trachoma?

Ocular variola was fairly common in the Asian subcontinent. Severe conjunctivitis was common in patients with smallpox, and corneal lesions developed in ≈7% of unvaccinated patients (*6*). Dixon reported that corneal lesions were most common in North Africa in patients with trachoma (*10*). Actual pocks occurred in vascularized parts of the conjunctiva or sclera in which pannus occurred. Obvious protein–calorie malnutrition also seemed to be a risk factor. Blood vessels in the conjunctiva and sclera characteristic of trachoma or other types of serious conjunctivitis in tropical areas enabled variola virions to be deposited on parts of the sclera that are usually avascular.

We cannot say with confidence that ocular variola was considerably more common in areas where trachoma or vitamin A deficiency was rare because rates of this devastating complication are not well documented in Europe and the United States; however, ocular variola certainly occurred. If trachoma is a predisposing condition, ocular variola may be rare in Western industrialized nations if smallpox reappears.

What is the Mechanism for Joint Destruction by Variola Major?

Variola major resulted in destruction of large joints, particularly of the elbows and knees, in ≈2% of unvaccinated children. It seems likely that that this joint destruction was caused by infection of the joint space or compromise of the blood supply by a viral arteritis, rather than by an immune-mediated arthropathy (*11*). Could ST-246 or CMX-001 be injected directly into the joint space, and if so would it help? If the mechanism is predominately poor blood flow secondary to an arteritis, an antiviral drug might not eliminate it. Direct injection of an antiviral drug into the joint spaces might be useful if we could agree on clinical indicators of joint infection that would induce such a therapeutic approach.

Will Vaccination during the Early Incubation Period, with or without Antiviral Drugs, Prevent Disease?

It was unethical, and probably logistically impossible, to conduct controlled field trials of vaccination at various periods into the incubation period during the many years that smallpox was being eliminated by using vigorous surveillance and vaccination of immediate contacts. A Delphi technique poll of experienced field workers concluded that most experts believed that vaccination within 4 days of exposure would prevent smallpox (*12*). Analysis of old data from the United Kingdom showed that good protection resulted from postexposure vaccination (*13*). The dynamics of development of humoral and cellular immunity after vaccination also suggest that vaccination within 3 days after exposure would be successful (*14*).

ST-246 seems to be an effective antiviral drug (*2*,*15*). Preliminary animal data and limited human data suggest that giving ST-246 with vaccination greatly reduces the clinical manifestations of vaccinia but does not impede development of cellular or humoral immunity (*15*). However, it seems counterintuitive to give a drug that virtually eliminates poxvirus replication and vaccinia virus unless we can be sure it will not reduce the effectiveness of vaccination.

Where Should Patients with New Cases of Smallpox Be Treated?

Smallpox was a nosocomial disease (*16,17*). Often a patient who intially had no diagnosis was imperfectly isolated, despite having a high fever. The virus spread to other patients and to medical staff by close personal contact. The disease was considered by many experts to be most common in caregivers. In modern practice, 2 points should be considered when framing strategies for the use of hospitals during outbreak control. The first consideration is the use of therapies that may, although unproven, be of considerable value in reducing the case-fatality rate. These therapies include newly developed antiviral drugs, pressor therapy for shock, treatment for DIC, and efforts to control the cytokine cascade. The second consideration is the presence in modern hospitals of patients with HIV, iatrogenic immune suppression, and atopic dermatitis.

These patients might become severely ill if exposed to smallpox virus. Their immune conditions may make vaccination difficult or dangerous if they are exposed to smallpox virus.

Evidence has shown that protocols for isolation of patients with fever and an undiagnosed rash are not rigidly followed in many general hospitals (*18*). It might be better to bring medical care to patients in a remote location (e.g., motel or defunct hospital) than to risk spread of the disease in a hospital. Fairly sophisticated medical care can now be given at home or in remote locations (*19*).

What Would Be the Characteristics of a Smallpox Virus Created by Bioengineering?

Recreation of the smallpox virus from published genetic sequences (www.poxvirus.org) is theoretically possible. Inserting minor modifications into the genome of a well-characterized strain of vaccinia virus should be even easier. Orthopoxviruses are large, stable, DNA viruses that are fairly easy to manipulate genetically (*3*,*20,21*). Technologies needed for creating live poxviruses from a variety of genetic fragments are readily available (*3*,*20*). Some practical issues would be involved in such laboratory recreation, but a modern well-equipped viral genetics laboratory would have minimal difficulties.

The Soviet Union allegedly inserted genes from other pathogens into variola major virus (*21*). Researchers working with mousepox virus have created a recombinant virus capable of escaping the effects of prior immunization with vaccinia virus (*22*).

Such work has obvious ethical problems, but would creating live smallpox viruses be something terrorists would really want to do? Although most Western nations have mechanisms for controlling smallpox outbreaks, most third-world nations do not. The experience with severe acute respiratory syndrome showed that even in the absence of a vaccine or antimicrobial drug, diseases spread by respiratory secretions can be controlled by vigorous identification and isolation of patients (*23,24*). If widespread transmission occurs of a newly created or genetically modified variola major virus, it is highly likely that it would spread to third-world nations, including homelands of terrorists. The resulting devastation would create a major public relations setback to terrorists. This likely blowback should inhibit the motivation of terrorists to recreate the variola virus or enhance its pathogenicity.

How Much Vaccine is Available, and How Should it Be Used?

The United States has ample supplies of second-generation and third-generation vaccines (*25*). The second-generation vaccine is ACAM2000 (Acambis, Cambridge, MA, USA), which is a plaque-purified distinct strain of New York City Board of Health vaccinia virus grown by using modern cell lines rather than the skin of calves. It produces reactions and immune responses similar to those of first-generation Dryvax vaccine (Wyeth Laboratories, New York, NY, USA) (*26*). Second-generation vaccines can be diluted 1:10 and still give excellent results. The third-generation vaccine is Immvamune (Bavarian Nordic, Kvistgaard, Denmark), a strain of modified vaccinia Ankara (MVA), which has been extensively tested for safety and protects animals from orthopoxvirus challenge (*1*). Two injections of MVA produce humoral and cellular immunity similar to that produced by first-generation and second-generation vaccines. Although a live virus, MVA does not replicate in human tissues and does not have the same risk of adverse reactions as first-generation and second-generation vaccines. It has been tested in patients with atopic dermatitis and in patients with HIV infections with T-cell counts >250 cells/mL (*1*).

Third-generation vaccines such as MVA may not be optimal for outbreak control. MVA is frozen, and thus must be thawed in the field (the manufacturer is developing a freeze-dried formulation). It requires syringes and needles and must be administered by someone trained to give injections. First-generation and second-generation vaccines are lyophilized and can be reconstituted and administered in the field with bifurcated needle scarification by persons with minimal training. Because MVA does not produce a visible lesion or scar, rapid determination of who has been already vaccinated is difficult. Optimal immunity with MVA requires 2 injections. In contrast, single injections are fully protective for first-generation and second-generation vaccines.

In the absence of a perfect animal model for smallpox, and because it is impossible to test these vaccines against smallpox, we have only laboratory evidence of their efficacy. Given these limitations, third-generation vaccines may be best for persons who anticipate possible exposure, such as military personnel or laboratory personnel working with orthopoxviruses. In an actual outbreak, ACAM2000 should be used for field vaccination. However, its use increases the risk for development of progressive vaccinia or eczema vaccinatum; these adverse events would then need to be treated with ST-246.

WHO is planning on creating a modest real, and substantial virtual, stockpile of vaccines. There are ≈60 million doses of vaccine in this stockpile and plans to increase it to ≈200 million doses. How much of this stockpile should be second-generation or third-generation vaccines that may not yet have been licensed widely? What should be the rules for release of such vaccines from the stockpile? Widespread use of first-generation or second-generation smallpox vaccines in era of AIDS and iatrogenic immune suppression by cancer chemotherapy or for transplant surgery seems

unlikely unless there are actual cases of smallpox. In that situation, we might respond by using first-generation and second-generation vaccines with proven efficacies.

Should Existing Variola Viruses be Destroyed?

The WHO-approved research agenda with variola major virus has been largely fulfilled (*1*–*5*). Modern viral genetics may have rendered destruction of the 2 official remaining stores of the virus moot because the virus can be recreated with minimal technical difficulty (vide supra). The US Institute of Medicine has issued a report that outlines robust scientific arguments for retaining the stocks of live virus (*27*).

Discussions about destruction of the remaining variola major virus stocks should not be limited to the scientific points set forth in the US Institute of Medicine report (*27*). Ethical, political, and public relations issues would be involved in recreating the smallpox virus. Retaining existing stores of live variola major virus has similar ethical and political problems. Terrorists or rogue states that have other weapons of mass destruction might see our possession of smallpox virus as a justification for their own development of a bioterrorist arsenal. If known official stocks are destroyed, then we will know that any new cases of smallpox are the result of deliberate maligning activities. Thus, as long as Russia and the United States possess the virus, we have lost the moral high ground. The known stocks of the virus ought to be destroyed, as has been repeatedly requested by the World Health Assembly.

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etymologia

Variola
[və-riʹo-lə]

From the Latin for pustules or pox, possibly derived from varus, for pimple, or varius, for speckled. The earliest documented use of the word variola as a name for smallpox occurs in the 6th century, during the reign of the Byzantine emperor Justinian I. Referred to in the vernacular as simply "the pox" for many centuries, in the 16th century variola became known commonly as smallpox to distinguish the disease from syphilis, the great pox.

Vaccination
[vakʺsĭ-naʹshən]

From the Latin vacca, for cow. English physician Edward Jenner coined the term vaccination in 1796 to describe inserting pus from cowpox lesions into open cuts on human patients to prevent smallpox. The term now refers to any immunizing procedure in which a vaccine is administered.

Source: Hopkins DR. The greatest killer: smallpox in history. Chicago: The University of Chicago Press; 2002; Oldstone MB. Viruses, plagues, and history. New York: Oxford University Press; 1998; Tudor V, Strati I. Smallpox. Cholera. Tunbridge Wells (UK): Abacus Press; 1977.

Should Remaining Stockpiles of Smallpox Virus (Variola) Be Destroyed?

Raymond S. Weinstein

In 2011, the World Health Organization will recommend the fate of existing smallpox stockpiles, but circumstances have changed since the complete destruction of these cultures was first proposed. Recent studies suggest that variola and its experimental surrogate, vaccinia, have a remarkable ability to modify the human immune response through complex mechanisms that scientists are only just beginning to unravel. Further study that might require intact virus is essential. Moreover, modern science now has the capability to recreate smallpox or a smallpox-like organism in the laboratory in addition to the risk of nature recreating it as it did once before. These factors strongly suggest that relegating smallpox to the autoclave of extinction would be ill advised.

In 2011, the World Health Organization (WHO) plans to announce its recommendation regarding the final destruction of all known remaining smallpox virus stockpiles. Smallpox, an ancient human scourge of unparalleled destructive importance throughout most of recorded human history (Figure 1), is believed to have emerged in the Middle East some 6,000–10,000 years ago (*1*,*2*) from either camelpox or the gerbil-specific taterapox (*3*–*5*). It holds a status as one of the great killers in all human history, having produced the horrific deaths of up to 500 million persons in just the 20th century alone (*6*). At first glance, the answer to this conundrum—whether or not smallpox should be forever relegated to the autoclave of extinction—might seem an easy one. Beaten back by the Jenner vaccine first proposed in 1796, smallpox was finally declared eradicated in 1980, in one of the most profound public health achievements in human history. Since that time, WHO has made it generally known that they would like to see the elimination of all remaining variola stockpiles and made the United States and Russia the repository for all remaining stocks. At the 60th Annual World Health Assembly in 2007, the organization postponed the final decision for any recommended destruction deadline until their next meeting in 2011.

The last officially acknowledged stocks of variola are held by the United States at the Centers for Disease Control and Prevention and by Russia at the State Research Centre of Virology and Biotechnology. The US collection consists of 450 isolates of variola, while various authoritative sources place the number of specimens retained by Russia at ≈150 samples, consisting of 120 different strains (*7*,*8*), including several selected for their increased virulence

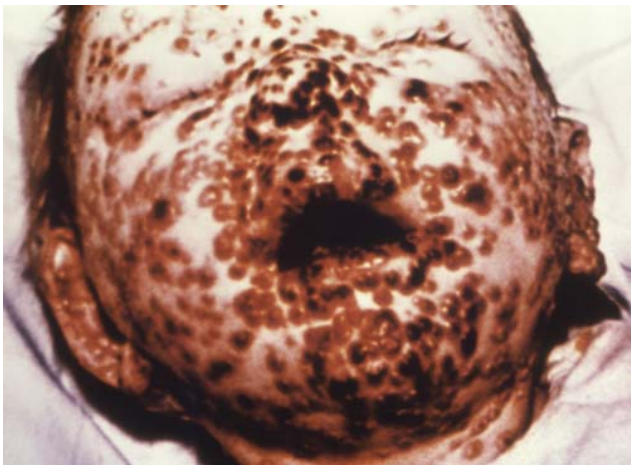

Figure 1. One-year-old child on day 10 of a smallpox infection; his face is covered with painful lesions that are beginning to scab. Photograph courtesy of the Centers for Disease Control and Prevention Public Health Image Library; by Charles Farmer, Jr., 1962.

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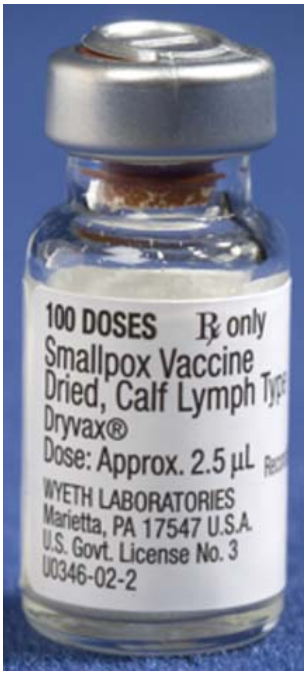

Figure 2. The no-longer-manufactured Wyeth vaccine that made possible the ultimate eradication of smallpox. Photograph courtesy of the Centers for Disease Control and Prevention Public Health Image Library; by James Gathany, 2002.

that were collected during the Cold War as potential biological weapons. The possibility that stolen smallpox cultures may already be in the hands of rogue states or terrorist organizations also remains an important subject of international concern.

Even though in 1980, then Secretary of Health and Human Services Louis Sullivan promised the destruction of US variola stockpiles within 3 years (*8*), this has not yet occurred in either the United States or Russia, and no actual recommendation for destruction has been issued by the World Health Assembly. To understand the reasons behind this apparent hesitance to once and for all eliminate from existence all remaining traces of the smallpox virus, one has to understand how the implications of this action have changed over the past several decades in a scientific world decidedly different from the one in which the idea of smallpox virus destruction was first proposed.

Currently, the only real benefit to destroying all known remaining stockpiles of variola in the world would be the elimination of the extremely unlikely possibility of unleashing a lethal epidemic due to the theft or accidental release of the virus from one of the remaining official stocks. In reality, this destruction would provide only an illusion of safety, and the drawbacks are many.

The prolonged existence of smallpox, combined with the important clinical implications of its high infectivity and mortality rates, suggests that the human immune system evolved under the disease's considerable evolutionary influence. In the past decade, for example, advances in immunologic research have suggested that the variola virus and its close relative and experimental surrogate, vaccinia, have a remarkable ability to substantially alter the immune response of its human host (*9*). Genomic and proteomic analysis and microarray surveys have demonstrated immunologic targets of smallpox that include, at minimum, several chemokines and their receptors, interleukin-8, interferon-γ, tumor necrosis factor–α, and the downstream target of receptor NFκB, and multiple components of the complement cascade (*10*–*15*). Although we are only just beginning to unravel the complex pathophysiology and virulence mechanisms of smallpox virus, experimental evidence with vaccinia has also demonstrated that many of the observed immunologic alterations produced by poxvirus infection persist long term and can be measured months or years after infection (*9*).

In addition, the evolutionary success of the CC-chemokine receptor null mutation, $CCR5^{\Delta 32}$, believed to have first appeared in northern Europe up to 3,500 years ago in a single person, is a good example of the importance of smallpox in human immune evolution (*16*). Today the mutation can be found in ≈10% of all those of northern European descent, preventing expression of the as-yet mostly inscrutable CCR5 receptor on the surface of many different subsets of immune cells. The huge success of this mutation is most likely because of the survival advantage it conferred by providing a marked resistance to smallpox (*16*–*18*). Notably, this same mutation confers nearly complete immunity to HIV. A recently published study suggests that the apparently sudden emergence and explosive spread of HIV may be related to the eradication of smallpox, postulating that widespread exposure to both variola and vaccinia (the virus that comprises the smallpox vaccine [Figure 2]) may have previously inhibited the successful spread of HIV (*19*). The immunologic mechanisms underlying this intriguing, and potentially useful, effect remain elusive. Thus, eliminating all known remaining smallpox stocks might hinder ongoing research in this direction.

The immune alterations produced by smallpox can serve as a window and guide to previously unappreciated immunologic mechanisms, the full understanding of which might lead to new therapeutic options for a host of diseases, both infectious and autoimmune. No one can yet be certain what role, if any, an intact variola virus might play in future research, and in providing important new insights into the human immune response as well as into the malevolence of this virus and related viruses. It is certain, however, that if the last remaining stockpiles are destroyed, the door to any possibility of future research employing the virus will be forever and irreversibly shut.

Finally, today's science is capable, through genetic manipulation, of re-creating a highly virulent smallpox-like virus from a closely related poxvirus or even from scratch. But perhaps what we should fear even more

is nature creating it for us, as it so efficiently did once before from the still-existent progenitors of variola. The possibility is certainly not unthinkable that nature could once again fashion smallpox from a near relative poxvirus or even create a new, smallpox-like human pathogen from a clinically similar but more genetically divergent zoonotic poxvirus, such as monkeypox. Several recent reviews have reported an increasing prevalence of human monkeypox since smallpox eradication and the cessation of vaccinia vaccination (*20*,*21*). The possible re-creation of smallpox by either natural or modern laboratory means would render moot any argument regarding the destruction of remaining stockpiles of smallpox virus in the mistaken belief that it would be for the benefit and protection of mankind.

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Parapoxvirus Infections of Red Deer, Italy

Alessandra Scagliarini,[1] Francesca Vaccari, Filippo Turrini, Alessandro Bianchi, Paolo Cordioli, and Antonio Lavazza

To characterize parapoxviruses causing severe disease in wild ruminants in Stelvio Park, Italy, we sequenced and compared the DNA of several isolates. Results demonstrated that the red deer isolates are closely related to the parapox of red deer in New Zealand virus.

The genus *Parapoxvirus* (family *Poxviridae*, subfamily *Chordopoxvirinae*) comprises several members: orf virus (OV), bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV), and parapox of red deer in New Zealand virus (PVNZ). PVNZ is responsible for a contagious pustular dermatitis in farmed red deer, with outbreaks reported only in New Zealand (*1*). Cases of parapoxvirus (PPV) pustular stomatitis were reported in wild ruminants in Stelvio Park in the Italian Alps during 2008. The affected animals had erosions and ulcers in the mouth, which led to death by starvation, particularly during the winter. Similar cases have also been described during 1992 in Finland and Norway in reindeer (*Rangifer tarandus*). Recently, the causative viruses of the clinical forms in reindeer were shown to be closely related to OV virus and PCPV, excluding the circulation of PVNZ in these countries (*2*,*3*).

To characterize the PPV agents causing severe disease in wild ruminants of Stelvio Park, we sequenced and compared the DNA of several isolates. Results showed that the viruses isolated from chamois (*Rupicapra rupicapra*) and ibex (*Capra ibex*) were closely related to OV, whereas the isolates from red deer (*Cervus elaphus*) grouped with PVNZ. Our findings provide new information about the diffusion of PPVs in wild ruminants and evidence that PVNZ is circulating outside New Zealand.

The Study

Cases of a severe contagious stomatitis were reported during winter 2008–09 in wild ruminants of Stelvio Park, Italy. Some affected animals were found dead with proliferative lesions, erosions, and ulcers on the lips and on the hard palate (Figure 1).

Samples collected for pathologic examination from 3 red deer, 2 chamois, and 1 ibex were submitted to Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna (Brescia, Italy) for identification of the causative agent of the disease. Viral particles, identified by morphologic features as PPV, were observed by negative stain electron microscopy in the material collected for pathologic examination from all the affected animals.

After injection into primary lamb keratinocytes (*4*), the viruses showed signs of cytopathic effect after at least 1 week. PCR was used to amplify the PPV *B2L* gene encoding a major envelop protein (*3*,*5*), and a specific amplification product has been obtained from all the isolates. To further characterize the viral DNA, we sequenced the PCR products and compared them with several *Chordopoxvirinae* homologue sequences of field and reference strains (Table). The PPV isolates from chamois and ibex were closely related to OV with 98.2%–99.3% identity at the nt level and 97.3%–98.4% identity at the aa level, compared with the reference strain OV NZ2. These results confirm little or no variations between PPVs that originate from different animal species and from different geographic areas as we and others already have reported (*2*–*6*).

Surprisingly, the *B2L* sequences of the 3 red deer isolates showed 100% identity with that of PVNZ RD86, suggesting that PVNZ could be the cause of the disease. To support this preliminary evidence, we further characterized the red deer viral strains. We amplified the vascular endothelial growth factor (*VEGF*) gene of the red deer isolates (*7*), which enabled us to obtain a specific amplification product from all 3 viral strains. The *VEGF* sequence of strain 348/09 showed 100% identity with PVNZ RD86, and strains 256/08 and 168/09 were 97.8% and 99.6% identical at the nt level and 96.55 and 98.8% identical at the aa level, respectively, which showed that multiple strains are circulating in Stelvio Park.

The classification of PVNZ as a new species of PPV originally was based on comparisons of restriction endonuclease digestion profiles and DNA hybridization analysis (*8*). Sequence analysis performed on the 2 genes of the 3 red deer isolates of Stelvio Park, confirmed the already reported genetic distance between PVNZ and the other PPV species (*3*,*7*). In particular, the phylogenetic analysis performed on the *B2L* gene showed that PVNZ sequences are closely related to BPSV (Figure 2, panel A), whereas the analyses conducted on the *VEGF* gene demonstrated a greater similarity between PVNZ, PCPV, and NZ-7–like VEGF variants (Figure 2, panel B). The latter analysis contributes to clarify the sequence relatedness between

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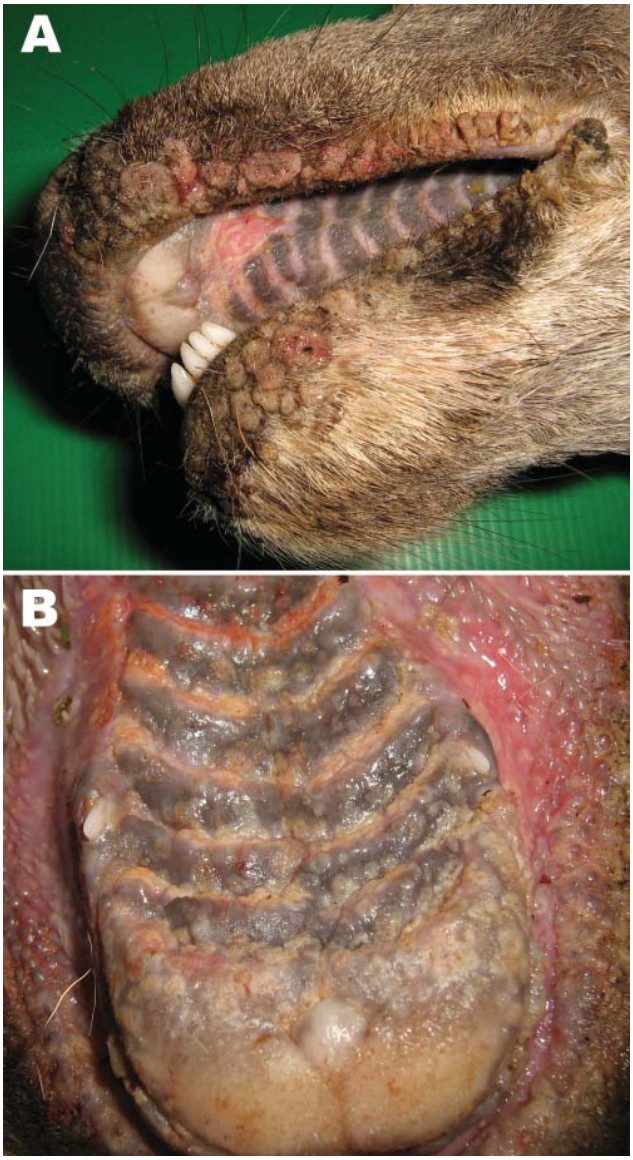

Figure 1. Papular stomatitis in a red deer. A) Proliferative lesions on the lips; B) erosions, vesicles, and ulcers in the mouth.

the 3 PPV species at the level of this gene, which further supports the hypothesis that PVNZ *VEGF* could have been acquired by natural recombination (*7*).

Conclusions

We demonstrated that the outbreaks of papular stomatitis in wild ruminants from Stelvio Park resulted from different PPV species: OV is the causative agent in chamois and ibex, and PVNZ caused the disease in red deer. Our results confirm that wild ruminants are susceptible to OV (*4*). This additional observation seems to prove that viruses isolated from chamois and ibex represent an adaptation of the same virus infecting domestic species rather than a separate viral species, despite the fact that the cause of contagious ecthyma in chamois is still considered a tentative species among the PPV genus by official taxonomy (*9*). In the cases reported here, no obvious connections existed between the disease in domestic and wild population, but transmission of PPV from sheep and goats to chamois and ibex cannot be excluded. PPVs are highly contagious and able to be transmitted either by direct contact between animals or indirectly by environmental contamination (*10*).

Table. Chordopoxviruses used for the phylogenetic analyses of the major envelope protein sequences*

Chordopoxvirus species	Original host	GenBank accession no.
PVNZ 168/09	Red deer	HQ239068
PVNZ 348/08	Red deer	HQ239070
PVNZ 256/08	Red deer	HQ239069
OV 257/09	Chamois	HQ239071
OV 373/08	Ibex	HQ239072
OV 485/09	Chamois	HQ239073
PCPV BO35	Bovine	AY453653
PCPV F00.128R	Reindeer	AY453657
PCPV F00120.R	Reindeer	GQ329669
PCPV F00.91.R	Reindeer	AY453658
PCPV VR634	Human	GQ329670
PCPV F99.177C	Bovine	AY453663
OV NZ-2	Sheep	U06671
OV IA82	Sheep	AY386263
OV F92.849	Reindeer	AY453659
OV Orf11	Sheep	AY453666
OV AICHI	Japanese serow	AB521165
OV SA00	Goat	AY386264
OV D1701	Sheep	AY453654
PVNZ RD86	Red deer	AY453655
BPSV Aomori	Bovine	AB044797
BPSV Chiba	Bovine	AB044798
BPSV V660	Bovine	AY453664
SPV	Seal	AF414182
CPXV ref strain BR	ATCC VR302	AF482758
MPXV	Human	AF380138
HPXV	Horse	DQ792504
VACV ref strain WR	ATCC VR1354	NC 006998
CMLV	Camel	AF438165
VARV	Human	L22579
SPPV	Sheeppox virus	AF199594
LSDV	Bovine	NC_003027
DPV	Mule deer	NC_006967
YLDV	Monkey	NC_002642
MOCV	Human	U60315
FWPV	Bird	AF198100

*PVNZ, parapox of red deer in New Zealand virus; OV, orf virus; PCPV, pseudocowpox virus; BPSV, bovine papular stomatitis virus; SPV, seal poxvirus; CPXV, cowpox virus; ATCC, American Type Culture Collection; MPXV, monkey poxvirus; HPXV, horsepox virus; VACV, vaccinia virus; CMLV, camelpox virus; VARV, variola virus; SPPV, sheeppox virus; LSDV, lumpy skin disease virus; DPV, deerpox virus; YLDV, Yaba like disease virus; MOCV, molluscum contagiosum virus; FWPV, fowlpox virus.

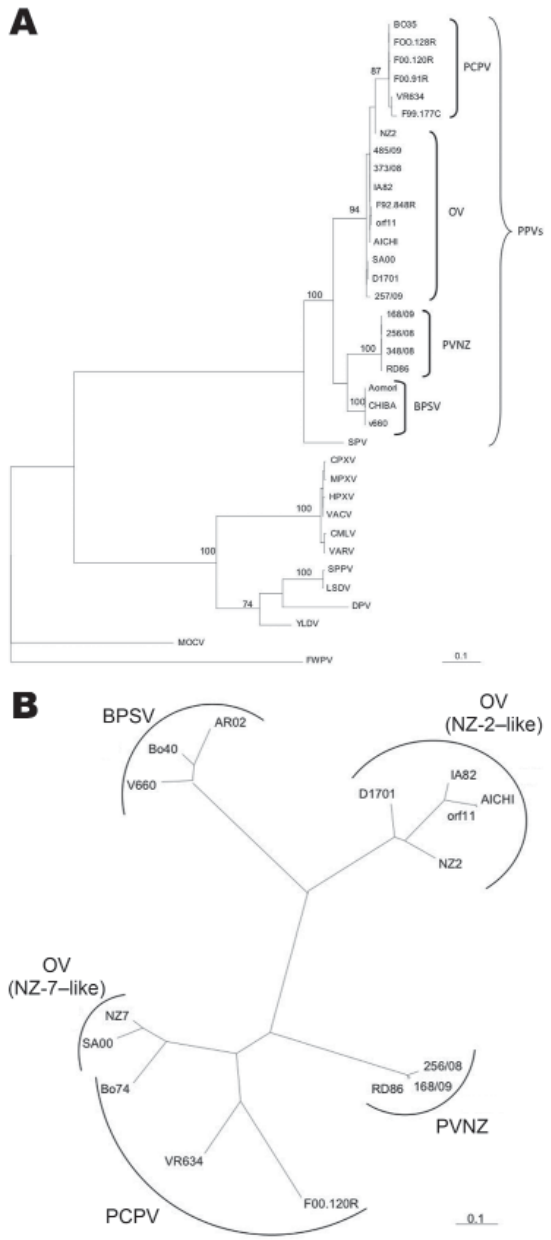

Figure 2. A) Phylogenetic tree of chordopox virus (Table) calculated from the deduced amino acid sequences of the major envelop protein gene. Chordopox virus sequences were edited to correspond to the amino acid sequences of parapoxviruses and aligned by using ClustalW (www.ebi.ac.uk/clustalw). Analyses were performed by using PHYLIP version 3.69 (distributed by J. Felsenstein, University of Washington, Seattle, WA, USA) and the maximum-likelihood method. Numbers on the nodes show the percentage of bootstrap calculated for 1,000 replicates. B) Phylogenetic tree based on the amino acid sequences of the parapoxviral vascular endothelial growth factor (*VEGF*) gene sequences. The genetic distance was estimated by using the Jones-Taylor-Thornton model of the program PRODIST and the phylogenies by using the Fitch-Margoliash method of FITCH (www.phylip.com). Tree was constructed by using PHYLIP version 3.69. Strain 348/08 is not shown because it shares 100% identity with RD-86. Scale bars indicate amino acid substitutions per site.

The genetic characterization of the strains isolated from red deer confirms the presence of PVNZ in Italy. This virus was first noted in New Zealand in 1986 (*1*). Red deer were brought into New Zealand from Europe around 1850, and PVNZ has been speculated to have been introduced from the old continent (*8*). Until now, the disease has never been reported outside New Zealand.

The clinical signs described in New Zealand red deer are generally mild, with lesions limited to the skin and to the epithelial surface of the growing antlers (velvet). In the cases reported here, the disease was severe enough to cause the deaths of the animals. We cannot exclude that mild forms also can occur in wild red deer, and that only the most severe cases of the disease might have been brought to our attention; for this reason, additional data are needed to elucidate the clinical features of the disease in Italy. No information is available about the possibility of PVNZ natural transmission to domestic species, but inoculation of the virus into OV-naive sheep produced milder lesions than did OV (*8*). Given the genetic similarity between the PPVs that are infecting red deer and cattle, ecologic studies should be designed to evaluate the susceptibility of these animal species, respectively, to PCPV, BPSV, and PVNZ.

Most PPVs are transmissible to humans, and these infections share clinical manifestations and exposure risks with other, potentially life-threatening zoonoses (*11*). The transmission of PPVs from deer to humans already has been reported (*12,13*); for these reasons, we cannot rule out that PVNZ could be transmitted to humans as a consequence of wildlife activities and manipulation of carcasses.

Dr Scagliarini is an associate professor at the Department of Veterinary Medical Science, University of Bologna and teaches in the Faculty of Veterinary Medicine. Her research interests are poxviruses and evaluation of the efficacy of antiviral compounds of synthetic and natural origin.

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Bacterial Meningitis and *Haemophilus influenzae* Type b Conjugate Vaccine, Malawi

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A retrospective database review showed that *Haemophilus influenzae* type b conjugate vaccine decreased the annual number of cases of *H. influenzae* type b meningitis in children in Blantyre, Malawi. Among young bacterial meningitis patients, HIV prevalence was high (36.7% during 1997–2009), and pneumococcus was the most common etiologic agent (57% in 2009).

Acute bacterial meningitis (ABM) is a major cause of illness and death in children in sub-Saharan Africa (*1*,*2*). *Neisseria meningitidis* is the most common cause of ABM in the meningitis belt (sub-Saharan Africa), and *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) are the most common causes in southern and eastern Africa (*1*–*4*). Of 114 case-patients with meningitis and positive cerebrospinal fluid (CSF) cultures who came to the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi (southeastern Africa), during 1996–1997, more than half of these cases were caused by *S. pneumoniae*, Hib, or *Salmonella* spp. (*5*).

In February 2002, Malawi introduced Hib conjugate vaccine in a pentavalent formulation that includes vaccine against diphtheria, pertussis, tetanus, and hepatitis B. There was no mass campaign or catch-up program. This vaccine is given routinely to patients at 6, 10, and 14 weeks of age; vaccination coverage has been ≈90% since 2002 (http://apps.who.int/immunization_monitoring/en/globalsummary/countryprofileresult.cfm). Incidence of Hib meningitis decreased but the long-term effect of the vaccination program remains unclear (*6*). We examined the effectiveness of Hib conjugate vaccine by conducting a retrospective database review of children with ABM who came to QECH in Blantyre, Malawi during 1997–2009.

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The Study

QECH is the district hospital for Blantyre District and a major referral center serving southern Malawi. We aggregated data from 3 studies of childhood ABM at QECH, where patients 2 months to 15 years of age with suspected ABM were treated (*7*; E.M. Molyneux, unpub. data). A patient with ABM was defined as a person whose CSF sample at the time of hospital admission contained ≥100 leukocytes/high-power microscopic field or demonstrable organisms by Gram stain or culture.

Data collection began in July 1997 and continued through December 2009. We multiplied the observed number of cases in 1997 by 2 to estimate the total number of cases for 1997. No data were collected during October 2000–October 2001; data from 2001 were excluded from analysis. Total number of cases in 2000 was estimated by multiplying the observed number of cases in 2000 by 1.25. *S. pneumoniae* and Hib had mild seasonal variations that did not affect the results, and we conclude that this estimation enables valid comparisons. We did not ascertain vaccination status of study participants.

Data were analyzed by using STATA 10.1 SE (Stata Corp LP, College Station, TX, USA). A 2-tailed t-test was used to compare mean case counts, and 2-tailed *z* tests were used to compare proportions. All studies were reviewed and approved by the College of Medicine Research and Ethics Committee in Blantyre, Malawi.

CSF samples were obtained aseptically before administration of antimicrobial drugs, labeled, and immediately sent to the laboratory. These specimens were cultured onto blood and chocolate agar plates and incubated at 37°C for 72 hours. Isolates were identified by using standard procedures (*8*). Commercial slide agglutination tests were used to serotype *H. influenzae* isolates (MAST Diagnostics, Bootle, UK). If CSF specimens were culture negative and gram negative after 2 days, they were tested for 5 common bacterial antigens (Hib, *S. pneumoniae*, *N. meningitidis*, group B streptococci, and *Escherichia coli*) by using latex agglutination reagents (Murex, Kent, UK) according to the manufacturer's instructions.

Serum samples were tested for HIV by using ≥2 of the following tests: Serodia-HIV particle agglutination (Fujirebio Inc., Tokyo, Japan and Mast Diagnostics), HIVSPOT (Genelabs Diagnostics, Singapore), Determine-HIV (Abbott Laboratories, Abbott Park, IL, USA), and Capillus-HIV (Cambridge Diagnostics, Galway, Ireland). Discordant test results were confirmed by using a third test or PCR for HIV. Children <15 months of age with positive antibody test results had their serostatus confirmed by PCR.

There were 1,740 children with bacterial meningitis at QECH during 1997–2009. Their ages ranged from 2 months to 15 years (median 18 months, mean 42 months).

One fourth of the children were <6 months of age and 53.8% were boys.

HIV serostatus was available for 1,486 patients; of these patients, 36.7% were HIV seropositive. This proportion increased from 30.4% (207/680) during 1997–2002 to 42% (336/801) during 2003–2009 (p<0.0001). The proportion of seropositive patients was similar for both sexes (girls, 271/696 [38.9%]; boys, 270/781 [34.6%]; p = 0.08). HIV test results were equivocal for 1 patient who was excluded from further analyses of HIV serostatus. On the basis of projections, ≈111,510 children <15 years of age were HIV seropositive in Malawi in 2009 (*9*). Census data show that there were 6,749,800 children <15 years of age in Malawi in 2009 (*10*). We estimate that 1.65% of children in Malawi are HIV seropositive. The proportion of HIV-seropositive children was significantly higher in our study population than in the general population (p<0.0001).

The number of annual cases for each causative agent of ABM changed dramatically during 1997–2009 (Figure). Before Hib vaccine was available (1997–2002), Hib was responsible for 53.2 annual cases of bacterial meningitis. After introduction of the vaccine, mean number of annual cases decreased to 9.7 (p<0.0001; Table). Mean age of these patients increased from 14 months (range 2–96 months, median 9 months) during 1997–2002 to 32 months (range 2–120 months, median 21 months) during 2003–2009 (p<0.0001).

The most prevalent cause of bacterial meningitis each year was *S. pneumoniae*. No change was observed in mean number of annual cases after introduction of Hib vaccine (1997–2002, mean 74.6 annual cases vs. 2003–2009, mean 78.1 annual cases; p = 0.66). The proportion of patients co-infected with pneumococcal meningitis and HIV increased from 41.5% in 1997–2002 to 49.6% in 2003–2009 (p = 0.03), and the proportion of patients co-infected with Hib meningitis and HIV did not change after introduction of conjugate vaccine (1997–2002, 43/204 [21.1%], vs. 2003–2009, 14/56 [25%]; p = 0.53). During 2003–2009, 7 (12.5%) of 56 Hib cases were in children <14 weeks of age. We observed a decrease in the mean number of annual cases caused by *N. meningitidis* during 2003–2009 compared with 1997–2002; this organism has caused <10 cases each year since 2002.

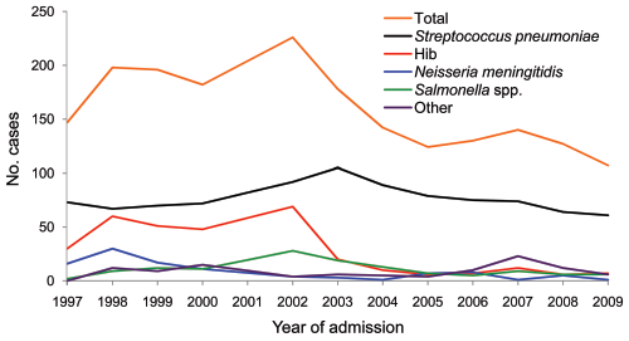

Figure. Annual number of cases of culture-positive bacterial meningitis in children, Queen Elizabeth Central Hospital, Blantyre, Malawi, 1997–2009. Data from 2001 are excluded. Hib, *Haemophilus influenzae* type b; Other, *Klebsiella* spp., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Brevundimonas vesicularis*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *H. influenzae* type C, *H. influenzae* not typed, group B streptococci, group A streptococci, and other species.

Conclusions

Cases of Hib meningitis in children decreased substantially in Blantyre in the postvaccination era but causes of residual disease remain unclear. We hypothesize that Hib meningitis affects mainly those not fully immunized, those with HIV, and those vaccinated before introduction of pentavalent vaccine. However, without data on vaccination status of participants, we cannot address this hypothesis and recommend more intensive research. These results complement and extend those of a previous study and provide evidence of ongoing effectiveness of the Hib conjugate vaccination program in a population with high HIV seroprevalence (*6*). Hib conjugate vaccine is not equally effective among HIV-positive patients, and the

Table. Causes of bacterial meningitis among patients at Queen Elizabeth Central Hospital, Blantyre, Malawi 1997–2009

Culture organism	No. cases (annual mean ± SE) 1997–2002*	2003–2009	p value
Streptococcus pneumoniae	373 (74.6 ± 4.83)	547 (78.1 ± 5.69)	0.66
Haemophilus influenzae type b	266 (53.2 ± 5.51)	68 (9.7 ± 1.91)	<0.0001
Neisseria meningitides	78 (15.6 ± 4.27)	26 (3.7 ± 1.12)	0.0106
Salmonella spp.†	62 (12.4 ± 4.27)	65 (9.3 ± 1.91)	0.48
Other‡	40 (8.0 ± 2.70)	66 (9.4 ± 2.51)	0.71
No growth	100 (25.8 ± 3.40)	205 (25.1 ± 1.65)	0.85

*Excludes data from 2001.
†*Salmonella enterica* serovar Typhimurium (79), *S. enterica* serovar Enteriditis (22), *S. enterica* serovar Typhi (14), and other *Salmonella* spp. (9). These numbers reflect actual case counts. Numbers were adjusted for missing data.
‡Includes *Klebsiella* spp. (4), *Staphylococcus aureus* (5), *Staphylococcus epidermidis* (2), *Escherichia coli* (5), *Brevundimonas vesicularis* (1), *Pseudomonas aeruginosa* (2), *Streptococcus pyogenes* (6), *H. influenzae* type C (4), *H. influenzae* nontype b (6), group B streptococci (2), group A streptococci (1), and other species and unidentified species observed by using Gram staining.

high prevalence of HIV infection may be associated with persistence of Hib meningitis (*11*). Most Hib disease is seen as pneumonia, and estimates of Hib disease incidence based on meningitis data consequently underestimate the true incidence in the general population (*2*,*12*).

Pneumococcal meningitis occurred more frequently in HIV-seropositive children than in the general population, suggesting that HIV infection is a predisposing factor. This finding has been widely reported elsewhere (*13*). Approximately 65% of children in South Africa with pneumococcal meningitis were HIV seropositive; the larger proportion of HIV co-infection in this population may be caused by the higher HIV prevalence in children in South Africa (4.5% vs. 1.7%) (*14*). Pneumococcal meningitis remains the leading cause of ABM in children in Malawi and we strongly recommend introducing the pneumococcal conjugate vaccine to reduce the incidence of ABM in this population.

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Rapid Genotyping of Swine Influenza Viruses

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The emergence of pandemic (H1N1) 2009 virus highlighted the need for enhanced surveillance of swine influenza viruses. We used real-time reverse–transcription PCR–based genotyping and found that this rapid and simple genotyping method may identify reassortants derived from viruses of Eurasian avian-like, triple reassortant-like, and pandemic (H1N1) 2009 virus lineages.

Co-infection of influenza A viruses enables viral gene reassortments, thereby generating progeny viruses with novel genotypes. Such reassortants may pose a serious public health threat, as exemplified by the emergence of pandemic influenza (H1N1) in 2009 (*1*). Transmission of pandemic (H1N1) 2009 virus from humans to pigs has been reported (*2*–*5*). We recently identified a reassortment between pandemic (H1N1) 2009 virus and swine influenza viruses in pigs (*6*). These results emphasize the potential role of pigs as a mixing vessel for influenza viruses and the need for screening tests that can identify major reassortment events in pigs.

We previously developed 8 monoplex SYBR green–based quantitative reverse transcription–PCRs to detect all 8 gene segments derived from the pandemic (H1N1) 2009 virus or virus segments that are closely related to this lineage (i.e., neuraminidase [NA] and matrix protein from the Eurasian avian-like swine linage and polymerase basic protein [PB] 2, PB1, polymerase acidic protein [PA], hemagglutinin [HA], nucleocapsid protein [NP], and nonstructural protein [NS]) from triple reassortant swine linage (*5*). Using these PCRs, we identified swine viruses of atypical genotypes. However, with the exception of the HA-specific assay, the melting-curve signals of pandemic (H1N1) 2009 virus may be indistinguishable from the positive signals generated from its sister clade as indicated above. To differentiate between these closely related groups of viruses, we further optimized these assays by adding sequence-specific hydrolysis probes in the SYBR green assays.

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The Study

For this study, all SYBR green assays were modified from the previously described assays (*5*), with the exception of the reverse primers for the newly designed PB1 and NS segments (Table 1). The subtype H1N1 swine influenza viruses isolated in Hong Kong during the past few years were mainly derived from the Eurasian avian-like swine lineage (*6*,*7*). To generate more precise genotyping data for our ongoing surveillance, the NA segment–specific assay was specifically designed to react with the pandemic (H1N1) 2009 virus and a portion of Eurasian avian-like swine viruses that are circulating in southeastern China (online Technical Appendix Figure 1, www.cdc.gov/EID/content/17/4/691-Techapp.pdf). To avoid overlapping the emission spectrum of SYBR green, we labeled all pandemic (H1N1) 2009 virus–specific hydrolysis probes (Integrated DNA Technologies, Inc., Coralville, IA, USA) with cyanine 5 (Cy5) and Black Hole Quencher-2 dyes at their 5′ and 3′ ends, respectively (Table 1). To enable use of short oligonucleotide sequences without compromising the annealing temperature of these probes, we modified the probes with locked nucleic acids (*7*). RNA extraction and complimentary DNA synthesis were identical to the protocols described (*5*,*8*). One microliter of 10-fold diluted complimentary DNA sample was amplified in a 20-μL reaction containing 10 μL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the corresponding primer probe set (0.5 μmol/L each). All reactions were optimized and performed simultaneously in a 7500 Sequence Detection System (Applied Biosystems) with the following conditions: 20 s at 95°C, followed by 30 cycles of 95°C for 3 s and 62°C for 30 s. SYBR green and Cy5 signals from the same reaction were captured simultaneously at the end of each amplification cycle. The expected PCR results of virus segment derived from different swine viral lineages are shown in Table 2.

The dissociation kinetics of PCR amplicons were studied by a melting curve analysis at the end of the PCR (60°C–95°C; temperature increment 0.1°C/s). We also tested various probe and SYBR green concentrations under different PCR conditions. The condition described above gave the most robust and consistent DNA amplification (data not shown). We tested 31 human pandemic (H1N1) 2009 and 63 human seasonal influenza viruses (33 subtype H1N1, 30 subtype H3N2) as controls. As expected, all human pandemic influenza viruses were double positive (i.e., positive with SYBR green and Cy5) and all seasonal influenza samples were double negative in all 8 assays.

Table 1. Primer–probe sets selective for pandemic (H1N1) 2009 virus gene segments*

Segment	Primer and probe†	Sequence, 5′ → 3′‡
PB2	PB2-1877F§	AACTTCTCCCCTTTGCTGCT
	PB2-2062R§	GATCTTCAGTCAATGCACCTG
	PB2-2028RP	Cy5-AACTGTAAGTCGTTTGGT-BHQ2
PB1	PB1-825F§	ACAGTCTGGGCTCCCAGTA
	PB1-1023R	GAACCACTCGGGTTGATTTCTG
	PB1-863FP	Cy5-CCAAACTGGCAAATG-BHQ2
PA	PA-821F§	GCCCCCTCAGATTGCCTG
	PA-1239R§	GCTTGCTAGAGATCTGGGC
	PA-844FP	Cy5-CCTCTTTGCCATCAGC-BHQ2
HA	HA-398F§	GAGCTCAGTGTCATCATTTGAA
	HA-570R§	TGCTGAGCTTTGGGTATGAA
	HA-470FP	Cy5-CAAAGGTGTAACGGCA-BHQ2
NP	NP-593F§	TGAAAGGAGTTGGAACAATAGCAA
	NP-942R§	GACCAGTGAGTACCCTTCCC
	NP-872RP	Cy5-AGGCAGGATTTATGTG-BHQ2
NA	NA-163F§	CATGCAATCAAAGCGTCATT
	NA-268R§	ACGGAAACCACTGACTGTCC
	NA-248RP	Cy5-AGCAGCAAAGTTGGTG-BHQ2
M	M-504F§	GGTCTCACAGACAGATGGCT
	M-818R§	GATCCCAATGATATTTGCTGCAATG
	M-530FP	Cy5-ACCAATCCACTAATCAGG-BHQ2
NS	NS-252F§	ACACTTAGAATGACAATTGCATCTGT
	NS-345R	GCATGAGCATGAACCAGTCTCG
	NS-288FP	Cy5-CGCTACCTTTCTGACAT/BHQ2/

*PB, polymerase basic protein; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleocapsid protein; NA, neuraminidase; M, matrix protein; NS, nonstructural protein; Cy5, cyanine 5; BHQ2, Black Hole Quencher 2.
†Number represents nucleotide position of the first base in the target sequence (cRNA sense).
‡Locked nucleic acid–modified bases are underlined.
§Primers adapted from the assays as previously described (*5*,*6*).

To evaluate the sensitivity of the assays, we tested serial diluted plasmid DNA of the corresponding segments of influenza A/California/4/2009 virus as a standard. The fluorescent signals generated from the SYBR green reporter dye in all assays were highly similar to those previously reported (*5*), and the modified assays had a linear dynamic detection range from 10^2 to 10^8 copies/reaction (online Technical Appendix Figure 2). As expected, the threshold cycle values deduced from the Cy5 reporter signal were generally higher than those from the SYBR green reporter (online Technical Appendix Figure 2) (*9*). This finding can be partly explained by the nature of these 2 kinds of real-time PCR chemistries: a single Cy5 fluorophore of the hydrolysis probe was released from quenching for each amplicon synthesized while multiple SYBR green dyes bound to a single amplicon (*10*). After 35 PCR amplification cycles, the linear dynamic detection range of Cy5 signals generated from these reactions was 10^2 to 10^8 copies/reaction (data not shown). However, to avoid nonspecific SYBR green signals, we purposely limited the number of amplification cycles to 30.

Using these assays, we tested 41 swine virus isolates collected during January 2009–January 2010. In all 8 reactions, 10 pandemic (H1N1) 2009 virus samples transmitted from humans to pigs (*6*) were double positive (online Technical Appendix Figure 1, pink). In these assays, gene segments of another 31 swine isolates were either SYBR green positive/Cy5 negative (online Technical Appendix Figure 1, yellow) or double negative (online Technical Appendix Figure 1, green), indicating that these virus segments were derived from the sister clade of pandemic (H1N1) 2009 virus or other swine

Table 2. Summary of expected genotyping results of swine and human influenza viruses*

Virus	PB2	PB1	PA	HA	NP	NA†	M	NS
Pandemic (H1N1) 2009	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Swine Eurasian avian-like	– –	– –	– –	– –	– –	– +	– +	– –
Swine triple reassortant	– +	– +	– +	– +	– +	– –	– –	– +
Human seasonal subtypes H1 and H3	– –	– –	– –	– –	– –	– –	– –	– –

*PB, polymerase basic protein; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleocapsid protein; NA, neuraminidase; M, matrix protein; NS, nonstructural protein. Red symbols indicate pandemic (H1N1) 2009 virus–specific probe results; black symbols indicate SYBR Green results; gray shading indicates sister clade of pandemic (H1N1) 2009 virus for each virus segment.
†N2 and some of the N1 within swine Euarasian avian-like lineage are expected to be double negative in the NA test.

lineages (except NA), respectively. For example, the reassortant of pandemic (H1N1) 2009 virus (A/swine/Hong Kong/201/2010 [H1N1]) was double positive for NA, double negative for HA and matrix protein, and SYBR green positive/Cy5 negative for PB2, PB1, PA, NP, and NS (Figure; other data not shown). All genotyping results of the studied viruses were consistent with results of previous phylogenetic analyses (*5*,*6*), indicating that our modified probes and SYBR green assays can provide more accurate genotyping results. With these genotyping data, viruses with atypical positive signal patterns might suggest a novel viral reassortment event and can be highlighted for investigation with sequencing-based methods.

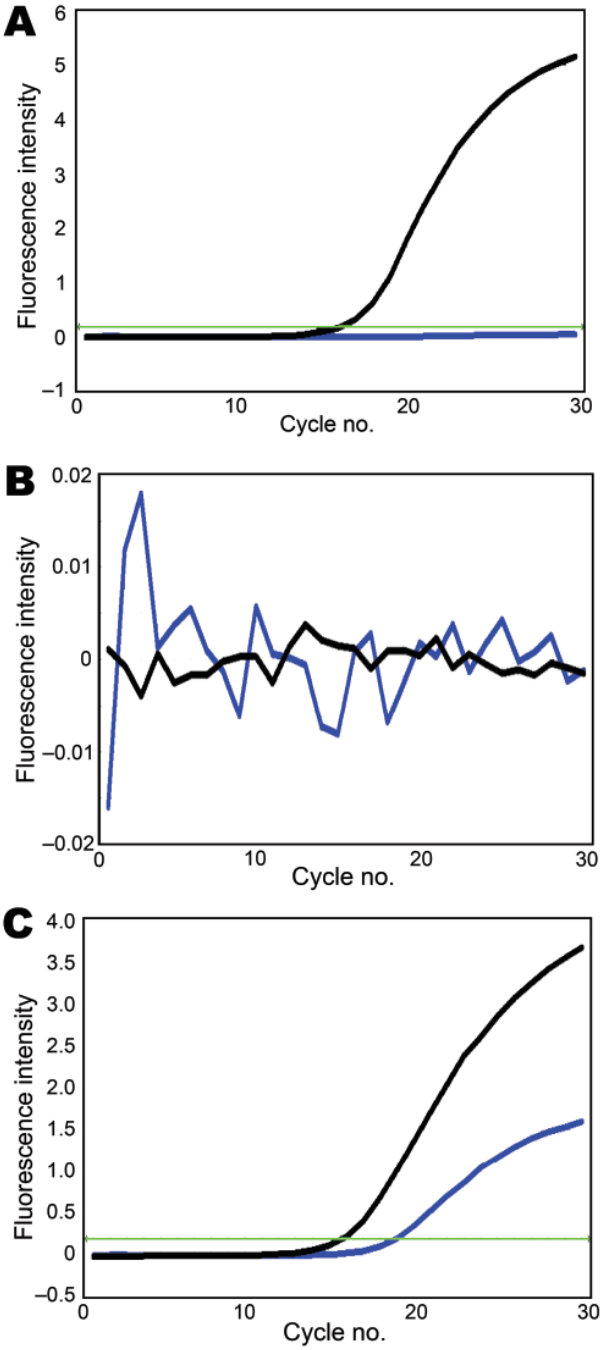

Figure. Genotyping of A) polymerase acidic protein, B) hemagglutinin, and C) neuraminidase segments of A/swine/Hong Kong/201/2010 influenza (H1N1) virus. Black line, amplification signal of SYBR green dye; blue line, amplification signal of cyanine 5 dye; green line, threshold level. The x-axis denotes the cycle number of a quantitative PCR assay, and the y-axis denotes the fluorescence intensity over the background.

To demonstrate the potential use of these assays in studying swine viruses circulating in other geographic locations, we tested 7 recent swine isolates (1 pandemic influenza subtype H1N1, 4 subtype H1N2, and 2 subtype H3N2) collected in the United States. Genotyping results agreed 100% with data deduced from sequence analyses (online Technical Appendix Table 1). We also analyzed all 436 contemporary (2008–2010) US swine virus segments available from the National Center for Biotechnology Information influenza virus sequence database. On the basis of the in silico analysis of sequences targeted by our primers and probes, 95% of the sequences (n = 413) are predicted to yield the expected results (online Technical Appendix Table 2).

Conclusions

The emergence of pandemic (H1N1) 2009 has highlighted the need for global systematic influenza surveillance in swine. Our results demonstrated that the addition of locked nucleic acid hydrolysis probes specific for pandemic (H1N1) 2009 virus into previously established SYBR green assays can help differentiate segments of pandemic (H1N1) 2009, Eurasian avian-like, and triple reassortant virus lineages. These assays might provide a rapid and simple genotyping method for identifying viruses that need to be fully genetically sequenced and characterized. They may also help provide better understanding of the viral reassortment events and viral dynamics in pigs. Although at present, genes derived from human seasonal viruses cannot be characterized with our modified assays, the performance of our assays warrants similar investigations for genotyping human influenza viruses.

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Molecular Discrimination of Sheep Bovine Spongiform Encephalopathy from Scrapie

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Sheep CH1641-like transmissible spongiform encephalopathy isolates have shown molecular similarities to bovine spongiform encephalopathy (BSE) isolates. We report that the prion protein PrP^{Sc} from sheep BSE is extremely resistant to denaturation. This feature, combined with the N-terminal PrP^{Sc} cleavage, allowed differentiation of classical scrapie, including CH1641-like, from natural goat BSE and experimental sheep BSE.

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle. TSEs are characterized by accumulation of an abnormal isoform of the host-encoded prion protein (PrP^{C}), termed PrP^{Sc}.

A novel human prion disease, variant CJD, was reported in 1995 and postulated to be caused by eating beef infected with BSE. Biologic and molecular analyses provided evidence that the same agent was involved in BSE and variant CJD (*1*,*2*). Evidence of sheep and goat susceptibility to BSE (*3*) and discovery of natural BSE infections in 2 goats (*4*,*5*) prompted the European Commission to increase the search for BSE infections in small ruminants. Although the BSE agent can be recognized by biologic strain typing in conventional mice (*2*), large-scale testing of small ruminants required molecular tests able to discriminate BSE from the most common TSEs of small ruminants.

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Molecular criteria used to discriminate BSE from scrapie are based on the low molecular weight of proteinase K–treated PrP^{Sc} (PrP^{res}) (*6*–*8*), a high proportion of the diglycosylated PrP^{Sc} (*1*,*6*,*8*), and poor or absent binding with antibodies directed at N-terminal epitopes (*8*–*10*). This last characteristic was fundamental in developing the discriminatory methods currently approved for surveillance in Europe (*11*).

The experimental scrapie isolate CH1641 reportedly shares molecular features with experimental sheep BSE (*7*), although lack of transmissibility of CH1641 to conventional mice in comparison to successful transmission of BSE provided evidence that CH1641 and BSE are caused by distinct prion agents. A few natural isolates have been described in sheep, showing molecular (*10*,*12*) and biologic (*13*) similarities to CH1641, and were named CH1641-like. Subtle pathologic differences were exploited to distinguish these CH1641-like isolates from BSE by immunohistochemical (*5*,*10*) and biochemical analyses by glycoform profiling (*8*,*10*). However, routine testing by using discriminatory Western blot (WB) methods does not easily distinguish CH1641 and CH1641-like isolates from BSE (*8*,*12*). We report 2 new CH1641-like isolates; analyze the conformational stability of CH1641-like isolates, BSE, and classical scrapie; and show that a reliable molecular differentiation of these 3 TSE sources is possible by an improved discriminatory WB method.

The Study

During 2009–2010, we analyzed conformational stability of PrP^{Sc} from sheep TSE isolates by using a conformational stability and solubility assay (CSSA) that we developed (*14*). We showed that CSSA could reveal strain-specified PrP^{Sc} conformational stability in sheep isolates because it enabled discrimination of Nor98 from classical scrapie isolates (*14*). Scrapie isolates had $GdnHCl_{1/2}$ values (the concentration of guanidine hydrochloride able to dissolve half the insoluble PrP^{Sc} aggregates in a brain homogenate) of 2.0 mol/L–2.3 mol/L; Nor98 isolates were less stable (1.3–1.4 mol/L GdnHCl). We thus sought to determine the conformational stability of PrP^{Sc} aggregates (online Technical Appendix, www.cdc.gov/EID/content/17/4/695-Techapp.pdf) derived from CH1641 and BSE strains (Table 1), including one (TR316211) of the few CH1641-like field isolates described so far (*10*,*12*,*13*). Two other CH1641-like isolates (99–454 and 99–321) were found in a retrospective analysis of sheep scrapie cases in France.

Classical scrapie included as control displayed a $GdnHCl_{1/2}$ value (2.2 mol/L) in the range of previously analyzed isolates. CH1641 (provided by N. Hunter, Institute for Animal Health, Edinburgh, Scotland) and CH1641-like isolates showed conformational stabilities close to classical

Table 1. Transmissible spongiform encephalopathy isolates analyzed by conformational stability and solubility assay*

Source	Identification no.	PrP genotype†	$GdnHCl_{1/2}$, mol/L ± SD‡
Natural isolates			
Scrapie	ES/8/10/2	ARQ/ARQ	2.19 ± 0.18
CH1641-like	99–454	VRQ/VRQ	2.00 ± 0.06
	99–321	VRQ/VRQ	2.41 ± 0.49
	TR316211	ARQ/ARQ	2.82 ± 0.08
Experimental samples			
CH1641	241/74	AxQ/AxQ	2.07 ± 0.05
Sheep BSE	301/16§	ARQ/ARQ	>4
	301/44§	ARQ/ARQ	>4
	302/90¶	ARQ/ARQ	3.8; >4; >4

*PrP, prion protein; $GdnHCL_{1/2}$, guanidine hydrochloride at a concentration able to dissolve half the insoluble aggregates in a brain homogenate; BSE, bovine spongiform encephalopathy.
†Amino acids at codons 136, 154 and 171.
‡Each sample was analyzed ≥3 times.
§Intracerebral transmission.
¶Oral transmission.

scrapie, with $GdnHCl_{1/2}$ values of 2.0–2.8 mol/L. In contrast, PrP^{Sc} from experimental sheep BSE (*15*) clearly showed higher conformational stability, with $GdnHCl_{1/2}$ values >3.8 mol/L (Table 1). These results suggest experimental sheep BSE might have a stronger resistance to denaturation than do most natural sheep scrapie isolates.

Because the discriminatory methods based on differential PrP^{Sc} N-terminal proteinase K (PK) cleavage (*11*) do not enable a clear-cut discrimination of CH1641-like from BSE (*12*), we investigated the potential of denaturation with GdnHCl as a further discriminatory strategy within the framework of the Istituto Superiore di Sanità discriminatory WB (*11*). To this aim, samples were untreated or treated with 3.5 mol/L GdnHCl before PK digestion and WB analysis with SAF84 and P4 monoclonal antibodies (Figure 1). This method was set up by analyzing representative scrapie, BSE, and CH1641 samples (Figure 1). As expected, BSE and CH1641 were poorly detected by P4, in contrast to classical scrapie. Treatment with 3.5 M GdnHCl, however, nearly abolished PK resistance of PrP^{Sc} from classical scrapie and CH1641, but not from sheep BSE, thus also enabling discrimination of CH1641 from BSE.

We then analyzed a larger set of samples (Table 2), including natural BSE in a goat (Figure 1). These experiments confirmed the higher resistance to denaturation of BSE samples, irrespective of the species, PrP genotype, and route of inoculation, compared with all other samples (Figure 1). When the antibody ratio and the denaturation ratio were measured and plotted as a scattergraph, classical scrapie, CH1641, and BSE isolates clustered into 3 distinct groups (Figure 2, panel A): 1) scrapie isolates displayed antibody ratios <2 and denaturation ratios were 0.02–0.13; 2) CH1641 samples had antibody ratios >2 and denaturation ratios were 0.06–0.29; and 3) BSE samples had antibody ratios >2, but denaturation ratios were >0.51.

Glycoform profiles, i.e., the relative proportion of diglycosylated, monoglycosylated, and unglycosylated PrP^{res} fragments, have also been reported as a discriminatory criterion for the identification of BSE in sheep (*8–10*), as well as when compared with CH1641 (*8*,*10*). With the Istituto Superiore di Sanità WB method (Figure 2, panel B), field scrapie isolates, including CH1641-like isolates, were characterized by a lower diglycosylated-to-monoglycosylated glycoform ratio (0.48:0.35–0.58:0.25)

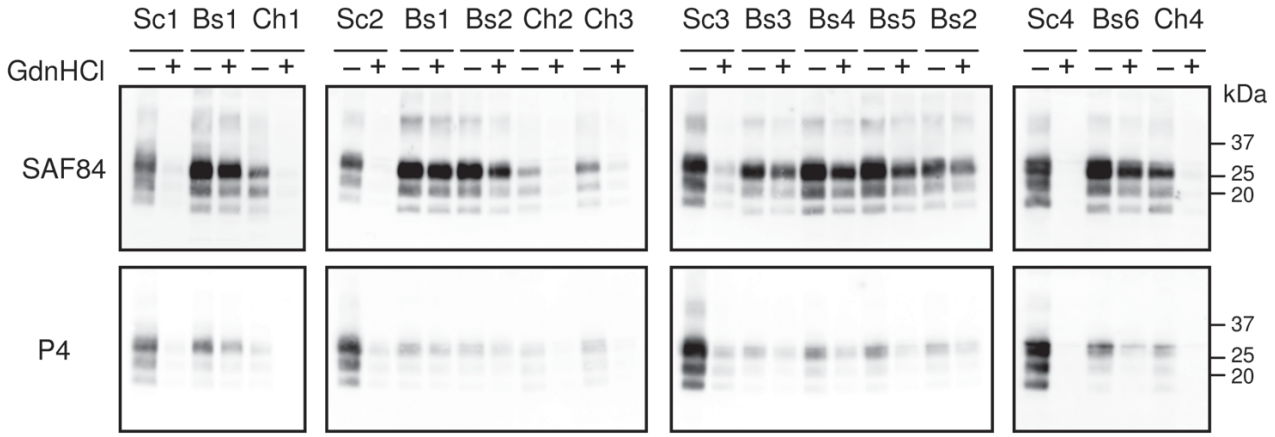

Figure 1. Representative Western blot showing the differential N-terminal proteinase K cleavage (monoclonal antibodies SAF84 vs. P4) and the susceptibility to denaturation of different transmissible spongiform encephalopathy isolates. Samples are indicated according to Table 2: classical scrapie isolates (Sc1, Sc2, Sc3, Sc4); experimental CH1641 (Ch1); CH1641-like isolates (Ch2, Ch3, Ch4); experimental sheep bovine spongiform encephalopathy by intracerebral transmission (Bs1) and oral transmission (Bs2, Bs3, Bs4, Bs5); natural goat isolate (Bs6). All samples were pretreated (+) or not treated (-) with 3.5 mol/L guanidine hydrochloride for 1 h at 37°C and then diluted to a final concentration of 0.35 mol/L guanidine hydrochloride, before digestion with proteinase K according to the Istituto Superiore di Sanità discriminatory method. Replica blots were probed with SAF84 (top) and P4 (bottom). Molecular weights are indicated on the right. GdnHCl, guanidine hydrochloride.

Table 2. Transmissible spongiform encephalopathy samples analyzed by discriminatory Western blot*

Source	Identification no.	PrP genotype†	Blot lane
Natural isolates			
Scrapie	ES16/10/10	ARQ/ARQ	Sc1
	ES16/10/11	ARQ/ARQ	Sc2
	ES16/10/12	ARQ/ARQ	Sc3
	ES12/10/1	ARQ/ARQ	
	ES12/10/2	ARQ/ARQ	
	ES12/10/3	ARQ/ARQ	Sc4
CH1641-like	99–454	VRQ/VRQ	Ch2
	99–321	VRQ/VRQ	Ch4
	TR316211	ARQ/ARQ	Ch3
Goat BSE	CH636		Bs6
Experimental samples			
CH1641	241/74	AxQ/AxQ	Ch1
Sheep BSE	301/16‡	ARQ/ARQ	Bs1
	301/44‡	ARQ/ARQ	
	302/87§	ARQ/ARQ	Bs3
	302/130§	ARQ/ARQ	Bs4
	302/64§	ARQ/AHQ	Bs5
	302/90§	ARQ/ARQ	Bs2

*Blot lanes shown in Figure 1. PrP, prion protein; BSE, bovine spongiform encephalopathy.
†Amino acids at codons 136, 154 and 171.
‡Intracerebral transmission.
§Oral transmission.

than sheep BSE (0.65:0.25–0.75:0.19) and the natural goat BSE (0.70:0.22).

Conclusions

Because the analysis of PrP^{Sc} from sheep prion isolates by CSSA showed an extremely high conformational stability of BSE samples, we improved the Istituto Superiore di Sanità discriminatory WB by including a pretreatment of brain homogenates with GdnHCl. Our results show that the combined use of 2 independent molecular features, N-terminal cleavage by PK and resistance to denaturation, could indeed differentiate classical scrapie and CH1641-like isolates from small ruminant BSE. Nonetheless, we observed some variability among the CH1641-like samples, either when analyzed by CSSA (Table 1) or by the discriminatory WB. As previously reported (*12*), the antibody ratios of some CH1641-like samples were close to the cutoff (Figure 2, panel A). Furthermore, the variable conformational stability observed by CSSA was also reflected in the denaturation ratios measured by discriminatory WB, with 2 CH1641-like samples showing a relatively higher resistance to GdnHCl than to all other scrapie samples. Because of the limited number of CH1641-like isolates, further studies are needed to evaluate their effective range of variability.

This variability may be disappointing for discriminatory purposes, but it may also hinder the possible presence of subtle PrP^{Sc} conformational (and possibly strain) variants in CH1641-like isolates. The biologic similarities of CH1641-like samples after transmission to ovine transgenic mice (*13*) and voles (U. Agrimi, unpub. data) were worth noting. Nevertheless, CH1641-like isolates induced a certain degree of PrP^{Sc} molecular variability in both rodent models (*13*; U. Agrimi, unpub. data), which might be related to the molecular variability in PrP^{Sc} extracted from sheep brain.

Although based on a limited set of samples, our study supports the notion that CH1641-like isolates can be convincingly discriminated from small ruminant BSE on

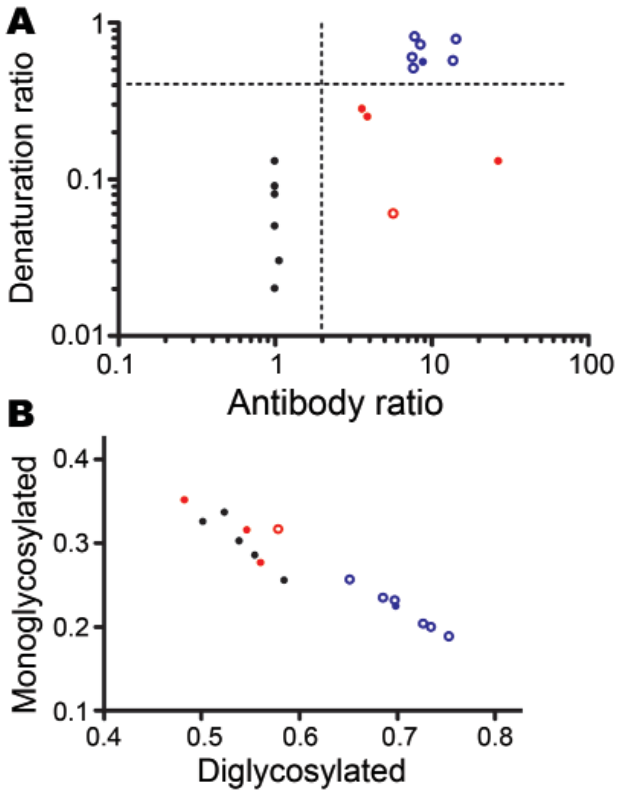

Figure 2. A) Scattergraph of antibody ratio and denaturation ratio obtained from each sample in Table 2, showing discrimination of scrapie, CH1641, CH1641-like, and bovine spongiform encephalopathy (BSE) samples. The antibody ratio is the SAF84/P4 ratio of the chemiluminescence signal relative to the SAF84/P4 ratio of the control scrapie loaded in each blot (online Technical Appendix, www.cdc.gov/EID/content/17/4/695-Techapp.pdf). The denaturation ratio, obtained from the SAF84 blot, is the ratio between the chemiluminescence signal with 3.5 mol/L and that with 0 mol/L. The vertical dashed line refers to the cutoff value of the antibody ratio, according to the Istituto Superiore di Sanità discriminatory Western blot (antibody ratio 2). The horizontal dashed line (denaturation ratio 0.4) shows the separation of BSE samples from all other transmissible spongiform encephalopathy sources. B) Scattergraph of proportions of diglycosylated and monoglycosylated PrPres bands from samples in Table 2. Results were obtained from guanidine hydrochloride–untreated samples in blots treated with SAF84. Classical scrapie samples are represented by black symbols, CH1641 by red symbols, and BSE samples by blue symbols. Filled symbols denote natural isolates and open symbols represent the experimental isolates.

molecular grounds. Furthermore, the high conformational stability of BSE, when compared with that in classical scrapie, Nor98, and CH1641-like isolates, suggests the potential of the new discriminatory WB here proposed for discriminating BSE from other known small ruminant TSEs.

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Ms Pirisinu is a PhD student in the Department of Veterinary Public Health and Food Safety at the Istituto Superiore di Sanità, Rome. Her research focuses on the molecular characterization of TSE strains in their natural hosts and in experimental animal models.

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Recent Clonal Origin of Cholera in Haiti

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Altered El Tor *Vibrio cholerae* O1, with classical cholera toxin B gene, was isolated from 16 patients with severe diarrhea at St. Mark's Hospital, Arbonite, Haiti, <3 weeks after onset of the current cholera epidemic. Variable-number tandem-repeat typing of 187 isolates showed minimal diversity, consistent with a point source for the epidemic.

On October 21, 2010, isolation of toxigenic *Vibrio cholerae* O1 from patients with severe diarrhea was confirmed by the National Laboratory of Public Health of the Ministry of Public Health and Population in Haiti (*1*). These cases indicated onset of epidemic cholera in Haiti and were followed by rapid spread of the disease throughout the country. Illness occurred in the setting of major disruptions of water and sewage facilities resulting from the January 12, 2010, earthquake and associated deficiencies in local public health infrastructure (*1*).

Before the current epidemic, cases of cholera had not been reported in Haiti since 1960 (*2*), and disease had not spread into Haiti during expansion of the El Tor pandemic into Latin America that began in Peru in 1991. However, toxigenic *V. cholerae* O1 are present along the US Gulf Coast (*3*,*4*) and in other coastal areas in the Western Hemisphere. In conjunction with ongoing public health activities in Haiti by the University of Florida, we analyzed fecal samples from patients early in the epidemic. Data obtained on *V. cholerae* strain diversity and gene content improved our understanding of the epidemiology of this outbreak.

The Study

Fecal samples from 19 patients were provided by staff at St. Mark's Hospital, Artibonite, Haiti, to University of Florida investigators on November 9, 2010, <3 weeks after onset of the epidemic. Microbial testing of samples was reviewed and approved by the University of Florida Institutional Review Board. Samples were directly plated on thiosulfate citrate bile salts sucrose agar and placed in alkaline peptone water for enrichment. Yellow colonies were identified by standard biochemical tests and confirmed by PCR for the outer membrane protein W gene (*5*).

V. cholerae O1 Ogawa was isolated from 16 of 19 samples. All isolates were El Tor biotype and had the El Tor type regulatory gene for phage lysogeny and the co-regulated pilus A gene identified by PCR (*6*,*7*). All isolates were newly identified altered El Tor that carried the classical cholera toxin B gene, as determined by mismatch amplification mutation assay–PCR (*8*).

To evaluate genetic diversity, we randomly chose ≤20 colonies (average 14.4) from each of 13 fecal samples that were culture positive without enrichment. A total of 187 colonies were typed by using multilocus variable-number tandem-repeat (VNTR) analysis as described (*9*–*12*). We used 5 loci (VC0147, VC0437 [the VC0436-7 intergenic region], VC1650, VCA0171, and VCA0283) reported by Ghosh et al. (*10*) and Choi et al. (*12*). Only 9 sequence types (STs) were identified (Figure); all were within 1 clonal complex, and each differed from the others by 1 allele. Type A (8,4,6,13,36) (Figure) was the dominant ST, present in 9 of 13 patients (Table). In 6 of these 9 patients, A was the only type identified. As reported (*10*), loci on the smaller chromosome had the greatest diversity (3 alleles for VCA0171 and 5 alleles for VCA0283) compared with no variation for VC0437 or VC1650 and only 1 change in VC0147. None of these STs has been reported in studies of strains from Bangladesh, India, Vietnam, or Mozambique (*9*–*12*).

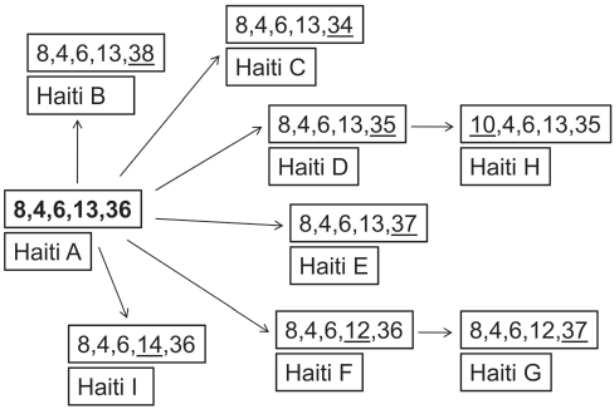

Figure. Relationship of *Vibrio cholerae* variable-number tandem-repeat sequence types from Haiti, 2010. Numbers represent number of repeats for the 5 alleles tested (VC0147, VC0436-7, VC1650, VCA0171, and VCA0283). **Boldface** indicates the ancestral sequence type; underline indicates alleles that have changed.

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Table. Distribution of *Vibrio cholerae* variable-number tandem-repeat sequence types among 16 patients with severe diarrhea, Haiti, 2010*

Pattern	Patient no. and distribution
A = 8,4,6,13,36	P1 = 20/20, P2 = 18/18, P4 = 4/19, P6 = 1/20, P7 = 15/20, P9 = 19/19, P12 = 11/11, P17 = 1/1, P18 = 19/19
B = 8,4,6,13,38	P6 = 1/20
C = 8,4,6,13,34	P4 = 7/19
D = 8,4,6,13,35	P4 = 4/19
E = 8,4,6,13,37	P6 = 18/20, P14 = 1/1, P15 = 20/20, P20 = 1/1
F = 8,4,6,12,37	P8 = 2/18
G = 8,4,6,12,36	P8 = 16/18
H = 10,4,6,13,35	P4 = 4/19
I = 8,4,6,14,36	P7 = 5/20

*Numbers represent number of repeats for the 5 alleles tested (VC0147, VC0436–7, VC1650, VCA0171, and VCA0283). A is the dominant sequence type, identified in 9 of 16 patients for whom variable-number tandem-repeat data were available. One sequence type was found for 6 of 9 patients for whom multiple isolates were typed.

Conclusions

Profound disruption of sanitary and public health infrastructure in Haiti resulting from the January 12, 2010, earthquake created an environment in which rapid spread of a disease such as cholera might be expected. However, because cholera had not been reported in Haiti since 1960, its sudden appearance raises questions about its origin, which has implications for understanding transmission pathways and potential for further spread.

Estuarine and freshwater/riverine (*13*) environments are well-recognized reservoirs for *V. cholerae* and show long-term persistence of epidemic strains in the absence of human cases. The US Gulf Coast is an excellent example of this environment, and periodic cholera cases occur there, likely linked to a common clonal strain that has a characteristic pulsed-field gel electrophoresis (PFGE) banding pattern (*14*). Cases along the Gulf Coast have intermittently appeared since 1978 (*3*). Indigenously acquired cholera cases linked to seafood consumption were reported in October 2005 in Louisiana in temporal association with Hurricanes Katrina and Rita (*4*).

When cholera appeared in Haiti in October 2010, cases were clustered along a 20-mile stretch of the Artibonite River, and 18 (67%) of 27 hospitalized patients reported drinking untreated water from the river or canals before illness onset, which is consistent with the river as a source of infection (*1*). Although these data are consistent with a limited origin for the epidemic, whether these strains represent a persistent clone in the river environment or a new strain in Haiti is unknown.

Epidemic strains of *V. cholerae* O1 and O139 show a high degree of genetic similarity, making it difficult to separate various subgroups by using standard molecular typing approaches such as ribotyping, PFGE, and multilocus sequence typing (*6*). PFGE types tend to change slowly and are useful primarily for distinguishing strains in different pandemics or between continental branches of pandemics (*11*,*14*). PFGE has shown that strains from Haiti are similar to strains from southern Asia and other regions (*1*). Sequences from 2 isolates from Haiti were most similar to El Tor isolates from Bangladesh in 2002 and 2008 (*15*).

In recent studies (*9*–*12*), VNTR typing has provided greater discrimination for ongoing or established epidemic O1 and O139 serogroups. Three loci (VC0147, VC0437, and VC1650) on the large chromosome were more stable (*9*–*11*) and are likely to be considered the best loci for estimating across distances, especially because of our observation of large differences in genotypes between locations 50 miles apart in rural Bangladesh (*9*).

Although genotype 8,4,6 has not been seen in other studies (*9*–*12*), genotype 9,4,6 was common in Dhaka (*11*) and associated with O1 Ogawa, the same serotype and biotype as strains from Haiti. In the absence of a more comprehensive global VNTR database, establishing a definite source for strains from Haiti on the basis of VNTR typing is not possible. However, our findings are consistent with those of others studies implicating southern Asia as the source for these strains on the basis of deletion/insertion data for the superintegron and the sulfamethoxazole/trimethoprim resistance integron island, and from analysis of single nucleotide polymorphisms, including those in the cholera toxin gene identified in the complete sequence of the strain from Haiti (*15*).

Our finding of only 9 STs differing at 1 allele among 187 colonies underscores the clonality of the strains from Haiti. Given the degree of diversity in STs among environmental strains in Bangladesh, including development of almost entirely different sets of multiple ST patterns among isolates from locations 50 miles apart, lack of diversity among isolates from Haiti would support the hypothesis that the epidemic in Haiti was caused by 1 clone that had little time to undergo diversification of STs expected of strains persistent in an environmental reservoir for extended periods.

Although altered El Tor strains have evolved only within the past several decades, which also argues against a long-standing environmental source (*8*), VNTR analysis, with its rapid molecular clock, can be used to test theories about the origin of the strain in Haiti and provide a unique opportunity to follow diversification of this clone over time and with human passage during an epidemic. This analysis also reinforces large diversity of strains seen in cholera-endemic regions, and the apparent likelihood that infections in these areas can be caused by simultaneous infections with multiple strains (*11*).

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Drug-Resistant Pandemic (H1N1) 2009, South Korea[1]

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Eleven patients with drug-resistant pandemic (H1N1) 2009 were identified in South Korea during May 2009–January 2010. Virus isolates from all patients had the H275Y mutation in the neuraminidase gene. One isolate had the I117M mutation. Of the 11 patients, 6 were ≤59 months of age, and 5 had underlying immunosuppressive conditions.

The Korea Centers for Disease Control and Prevention asked clinicians to report all patients with suspected cases of drug-resistant pandemic (H1N1) 2009 when these patients showed treatment failure for oseltamivir or had unusually prolonged viral shedding (defined as >5 days after the onset of symptoms) (*1*). We report nationwide surveillance data on the epidemiologic and clinical characteristics of patients infected with pandemic (H1N1) 2009 in South Korea.

The Study

From the first reported infected patient in May 2009 through January 2010, a total of 740,835 patients in South Korea were reported as having pandemic (H1N1) 2009 virus infection. A total of 225 patients (0.03%) died of disease related to pandemic (H1N1) 2009. During this period, physicians in local clinics and tertiary hospitals sent specimens from 67 patients who were suspected of having drug-resistant pandemic (H1N1) 2009 to the Korea Centers for Disease Control; 11 patients (16%) had drug-resistant virus (Figure). After confirmation of drug resistance, Epidemic Intelligence Service officers obtained clinical and epidemiologic data by medical record review and interviews with household contacts and attending physicians for all patients.

To investigate whether virus contained genetic markers associated with resistance to antiviral drugs, a conventional genotyping assay (sequencing) was performed, and neuraminidase (NA) and matrix 2 genes were sequenced. All 11 patients had virus with a histidine-to-tyrosine mutation at residue 275 of the NA protein (H275Y); 1 patient also had virus with an I117M mutation (Figure). Detailed molecular epidemiologic data and genetic characteristics of the isolates are described elsewhere (*2*).

Of the 11 patients, 6 were ≤59 months of age and 5 had underlying immunosuppressive conditions; only 1 patient was immunocompetent and >59 months of age (Table). Three patients from whom samples before and after treatment with oseltamivir were available showed evidence of having acquired the H275Y mutation during oseltamivir therapy. None of the 11 patients received oseltamivir chemoprophylaxis.

We also tested 100 persons who had contact with the 11 patients for possible transmission of drug resistance. Eight of 100 were confirmed as having been infected with pandemic (H1N1) 2009 virus before the 11 patients were infected. Influenza-like illnesses developed in the 11 patients a median of 2 days (range 1–7 days) after the 8 contact persons were confirmed as having pandemic (H1N1) 2009. Five of the 8 contact patients were children; none had an immunosuppressive condition or were given oseltamivir chemoprophylaxis before illness; and 7 of 8 were ≤59 months of age. Oseltamivir-resistance tests were not performed for these 8 patients because they all received oseltamivir therapy and their clinical symptoms resolved. Therefore, the possibility of transmitted resistance from contact patients was not demonstrated in this study.

All 11 patients were initially given the usual dose of oseltamivir (75 mg 2×/day in adults). After detection of oseltamivir resistance, treatment regimens were as follows: 3 patients were given high-dose oseltamivir (150 mg 2×/day in adults), 3 patients were given combination therapy (oseltamivir and amantadine; oseltamivir and peramivir; and oseltamivir, amantadine, and ribavirin, respectively); 3 patients were given zanamivir nasally; and 2 patients continued to receive oseltamivir.

Seven of the 11 patients had complications during treatment: 6 had viral or secondary bacterial pneumonia and 1 had acute respiratory distress syndrome (Table). Three patients died of pandemic (H1N1) 2009. Patient 3 died 15 days after confirmation of infection with pandemic (H1N1) 2009 virus and 10 days after the emergence of oseltamivir-resistant virus. Patient 4 died 15 days after confirmation of infection and 4 days after emergence of oseltamivir-resistant virus. Patient 8, who was infected with virus that had H275Y and I117M mutations, died 18 days after confirmation of infection and 4 days after the emergence of oseltamivir-resistant virus.

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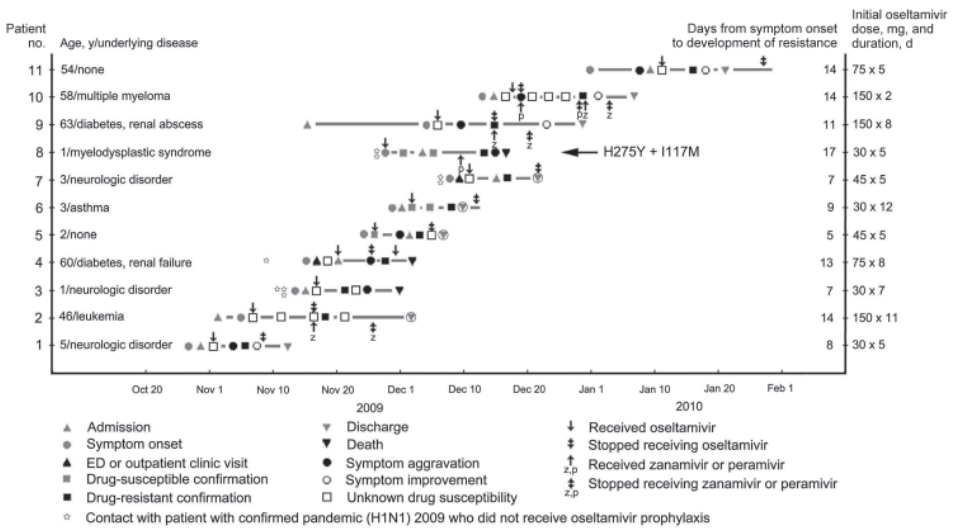

Figure. Clinical course and outcome of 11 patients with oseltamivir-resistant pandemic (H1N1) 2009, South Korea. Symptom aggravation was defined as influenza-related symptoms that worsened regardless of new infiltrations seen by chest radiography. Symptom improvement was defined as influenza-related symptoms (nasal stuffiness, sore throat, cough, myalgia, fatigue, headache, and fever) that were absent or mild. All doses of oseltamivir were given 2×/d. ED, emergency department.

Conclusions

Our nationwide surveillance of drug-resistant pandemic (H1N1) 2009 in South Korea indicated that most patients were children (≤59 months of age) or immunocompromised. All isolates had the H275Y mutation in the NA protein, and 1 isolate also had the I117M mutation in the same protein.

Our finding that oseltamivir resistance developed in immunocompromised patients is consistent with those of recent case reports that described development of oseltamivir resistance in immunosuppressed patients receiving this drug (*3*,*4*). A recent study in Australia reported that 4 of 32 adult oncology and hematology patients were infected with oseltamivir-resistant virus with the H275Y mutation (*5*). In 3 of our patients (patients 3, 5, and 11) drug-resistant isolates appeared ≤5 days after initiation of oseltamivir therapy. Thus, we suggest that physicians be alert to emergence of oseltamivir-resistant pandemic (H1N1) 2009, particularly if there is treatment failure with oseltamivir or prolonged viral shedding is evident.

More than half of our patients were ≤59 months of age, indicating that a younger age may be a risk factor for infection with drug-resistant pandemic (H1N1) 2009. Clinical trials have reported oseltamivir resistance in ≤5.5% of children with seasonal influenza (*6*). Explanations for the higher rate of drug resistance in children than in adults are that children have a more protracted course of influenza, longer viral shedding times, and higher viral titers (*7*). Another explanation might be that we cannot rule out suboptimal dosing of oseltmaivir in children, although World Health Organization dose standards were used (*8*) and all pharmacies were given instructions on emergency compounding of oseltmaivir.

Development of resistance appeared to be caused by sporadic mutations. We found no evidence of transmission. Availability of pretreatment and posttreatment samples indicated that resistance to oseltamivir developed during treatment in ≥3 patients. One case report (*9*), 1 report of a cluster of cases in Vietnam (*10*), and 1 outbreak in a hematologic ward (*11*) documented patient-to-patient transmission of oseltamivir-resistant virus. One of our patients was infected with virus that had a novel NA mutation (I117M). A previous study indicated that the I117V mutation in avian influenza virus (H5N1) was associated with low-level oseltamivir resistance (*12*). The I117M mutation of NA may contribute to oseltamivir resistance, but it is not clear whether isolates with these 2 NA mutations have higher levels of resistance or virulence than the H275Y NA mutant.

We found few patients with drug-resistant pandemic (H1N1) 2009 in South Korea. However, our study was based on the nationwide surveillance system for drug resistance among patients with suspected oseltamivir treatment failures, which is different from other surveillance systems, in which all isolated viruses are tested. Thus, we cannot report the prevalence of drug-resistant pandemic (H1N1) 2009 and risk factors for drug-resistant virus infection in South Korea. Further studies are needed to monitor oseltamivir resistance, especially in immunosuppressed or pediatric patients when treatment failure with oseltamivir or prolonged viral shedding occur. Additional studies are also needed to determine proper oseltamivir dosing for children.

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Table. Characteristics of 11 patients with oseltamivir-resistant pandemic (H1N1) 2009, South Korea*

Characteristic	Value
Median age, y (range)	5 (1–63)
Male sex	6
No. at high risk for influenza-related complications	
≤59 mo of age	6
Chronic medical disorders	9
Neurologic	3
Hematologic	3
Metabolic, including diabetes†	2
Pulmonary, including asthma	1
Renal†	1
Cardiovascular, excluding hypertension	0
Hepatic disorder	0
Immunocompromised‡	5
Median days from symptom onset to viral isolation (range)	2 (0–9)
Median days from symptom onset to development of resistance	8
No. hospitalized	11
Hospitalization duration, d, median (range)	15 (6–53)
Symptom duration, d, median (range)	17 (6–22)
No. with respiratory illness related to influenza	11
Viral pneumonia	6
Secondary bacterial pneumonia	2
Secondary bacterial pneumonia and acute respiratory distress syndrome	1
Reasons for requesting drug-resistance testing	
Treatment failure	9
Prolonged viral shedding	2
No. with co-infections§	3
Outcome	
Cured	8
Died¶	3

*Specimens were obtained from oropharyngeal (n = 7) and nasopharyngeal (n = 2) swabs, nasopharyngeal washings (n = 1), and brochoalveolar lavage fluid (n = 1).
†One patient had diabetes and chronic renal failure.
‡Patients who had underlying diseases such as HIV infection, malignancy, liver cirrhosis, or chronic renal failure, or patients receiving immunosuppressive treatment.
§Carbapenem-resistant *Pseudomonas aeruginosa* (patient 3), carbapenem-resistant *Acinetobacter baumanii* (patient 4), and penicillin-susceptible *Streptococcus pneumonia* (patient 10).
¶Of the 3 patients, 1 was infected with carbapenem-resistant *P. aeruginosa* (patient 3) and 1 was infected with carbapenem-resistant *A. baumanii* (patient 4).

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Seasonality of Cat-Scratch Disease, France, 1999–2009

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Cat-scratch disease is seasonal in the United States and Japan; but no data are available from Europe. To assess the seasonality of the disease in France, we analyzed lymph node biopsy specimens collected during 1999–2009. Most (87.5%) cases occurred during September–April and peaked in December.

Bartonella henselae is the causative agent of cat-scratch disease (CSD), the most common cause of lymphadenopathy in adults and children (*1*). Cats are the main reservoir of *B. henselae*, which is transmitted among cats by the *Ctenocephalides felis* flea (*2*). *Bartonella* organisms remain viable in flea feces, and transmission to humans results in inoculation of *B. henselae*–contaminated flea feces into the skin through a scratch (*3*). However, transmission of *B. henselae* from cats to humans through scratches is rare (*4*). In classic CSD, gradual regional lymph node enlargement is accompanied by a papule that develops in the scratch line after 3–10 days and persists from a few days to 2–3 weeks (*4*).

The link between seasons and CSD incidence has been described in the United States (*5*,*6*) and in Japan (*7*). However, because no data are available on seasonal variations of CSD in Europe, or in France, we studied lymph node biopsy specimens obtained January 1999–December 2009 from patients throughout France with suspected CSD.

The Study

Tissue specimens were sent to the National Reference Center (Marseilles, France) either frozen or in transport media. CSD was definitively diagnosed when a specimen was positive for 2 genes of *Bartonella* spp. (*1*). Total genomic DNA was extracted from samples by using a QIAamp tissue kit (QIAGEN, Hilden, Germany). Before 2005, PCR amplification and sequencing of the internal transcribed spacer (ITS) region and *pap31* gene were used for detecting *B. henselae* and thus confirming CSD (*1*). Beginning in 2005, real-time PCR to amplify the ITS region and *pap31* gene was used (*8*). For all assays, 2 sets of negative controls were used. DNA from *B. elizabethae* and *B. henselae* Houston-I was used as the positive control for the ITS region and the *pap31* gene, respectively (*1*). To exclude false-positive results, we performed a second independent extraction when false-positive or unexpected results were obtained. Results were confirmed by using PCR amplification and sequencing aimed at 16S rRNA gene (*8*).

Epi Info version 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA) was used for significance variations in the number of positive specimens between 2 consecutive months, nonconsecutive months, and seasons ($p<0.05$). The Mantel-Haenszel test or the Fisher exact test was used to test for significance.

We tested 1,849 lymph node biopsy specimens and identified *B. henselae* in 493. Positive and negative controls yielded the expected results in all tests. Positive CSD cases were plotted for each month (Figure 1) to identify seasonal distributions of CSD from 1999 through 2009. Monthly mean numbers of CSD were lowest from May through August, followed by significant increases during August–September ($p = 0.002$) and during November–December ($p = 0.01$). During December–January, the mean number of CSD cases decreased significantly ($p = 0.005$), then plateaued from January through March (Table). Cases decreased slightly in April, then decreased significantly during April–May ($p = 0.002$).

The odds of a CSD diagnosis based on lymph node biopsy was 9.2× higher in December, the month with the highest number of cases (92/493), than in July, the month with the lowest number of cases (12/493). The number of CSD cases was significantly higher in autumn (October–December) than in summer (July–September) ($p<0.0001$). Fewer cases were identified in winter (January–March) than

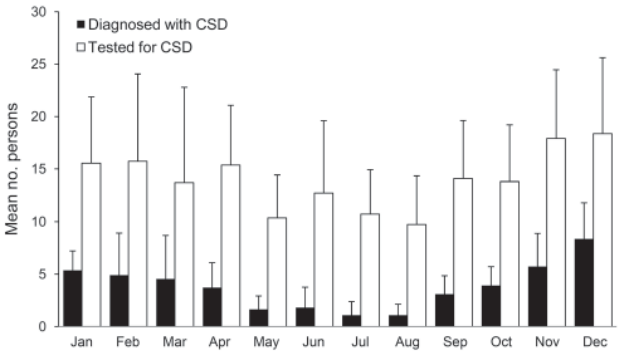

Figure 1. Mean numbers of patients tested for cat-scratch disease and for whom the disease was diagnosed, France, 1999–2009. Error bars indicate 95% confidence intervals.

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Table. Monthly number of patients with cat-scratch disease, France, 1999–2009

Month	Mean no. patients	Median no. patients	SD
January	5.4	6	1.86
February	4.9	6	4
March	4.6	2	4.2
April	3.7	4	2.4
May	1.6	1	1.3
June	1.8	1	1.9
July	1.1	1	1.3
August	1.1	1	1
September	3.1	3	1.8
October	3.9	4	1.8
November	5.7	5	3.2
December	8.4	7	3.4

autumn (p = 0.02), and cases decreased significantly from winter to spring (April–June) (p = 0.0001). The number of cases did not differ significantly from spring to summer (p = 0.06).

Conclusions

Our findings that the number CSD cases in France varies by season are similar to findings in Japan and the United States. In Japan, 64% of CSD occurred during September–December and peaked in November (*7*). In the United States, most CSD have occurred during the last 6 months of the year, with a peak in September (*9*). Moreover, the analysis of 3 US national databases indicated that most CSD cases have occurred during September–January, with peaks in November and December (*5*). On the other hand, 60% of admissions for CSD in children in the United States have occurred during July–October (*6*). The fact that the United States is a large country with diverse climates, whereas continental France has a more homogeneous climate, may explain the differences in seasonality.

The presence of *Ct. felis* fleas is essential for maintaining *B. henselae* infection within the cat population (*2*). Flea infestation is more frequent in bacteremic than in nonbacteremic cats, particularly in pet cats (*10*). After adult cat fleas parasitize a host cat, they feed on its blood and transmit *B. henselae*. Fleas go through 4 life cycle stages: egg, larva, pupa, and imago (adult) (Figure 2). Temperature and relative humidity are the 2 most essential factors for the successful reproduction, development, and survival of fleas (*11*). Cats reported to have been infested with fleas during the preceding 6 months were more likely than cats without fleas to be seropositive (*12*), and the seroprevalence of *B. henselae* is higher in the pet cat population in warm, humid climates than in cold, dry climates because *Ct. felis* fleas are more common in warmer climates (*10*). As a result, cats have more fleas during the summer and autumn months than in the other 2 seasons (*13*).

In Nancy, France, 53% of 94 stray cats were infected with either *B. henselae* or *B. clarridgeiae* (*14*). In Paris, Chomel et al. reported a *B. henselae* seroprevalence of 36% in 64 pet cats, of which 11% were *B. henselae* infected (*12*). Gurfield et al. determined that 16.5% of cats tested were *Bartonella* infected, and 41% were seropositive for *B. henselae* or *B. clarridgeiae* (*15*). Risk for *Bartonella* infection or seropositivity was higher in cats from multicat households and in cats adopted from the pound or from the street (*15*).

Feline sexual activity also may influence the seasonality of CSD. In the Northern Hemisphere, cat reproduction increases during spring and summer, and kittens stay with their mothers until they are 12–16 weeks of age. In addition, humans are more likely to acquire kittens during the autumn months. *B. henselae* infection appears to be more common in young cats (*10*), and infection decreases with the length of cat ownership (*15*). In addition, cats

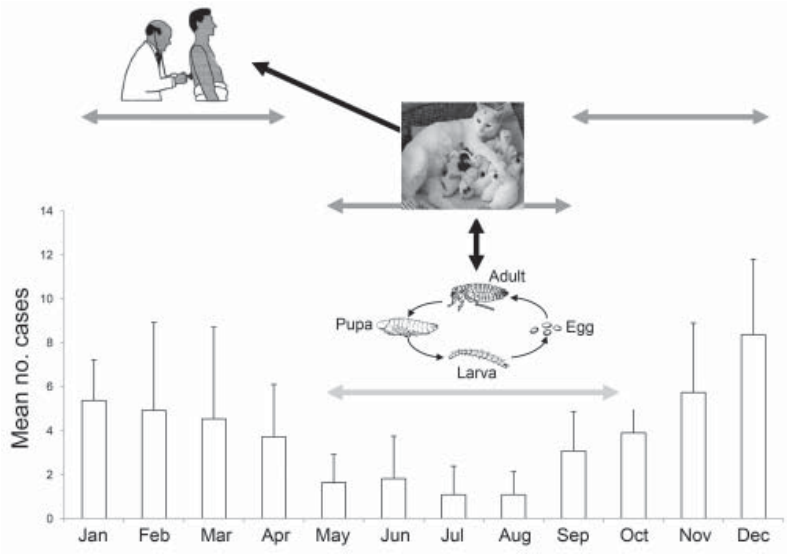

Figure 2. Explanation of cat scratch disease seasonality by seasonality of the birth of cats and the activity of their fleas, France, 1999–2009. Error bars indicate 95% confidence intervals.

encounter more fleas during summer and autumn, and transmission of *B. henselae* from cat to cat is facilitated during this period (*13*).

In conclusion, CSD is a seasonal disease in France with increased incidence in autumn, with peaks in December, and a decrease in spring. This pattern may be explained by seasonality in cat reproductive behavior, their fleas' activities, and the fact that during summer cats spend most time outside the house, whereas during autumn they stay indoors.

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Characteristics of Children Hospitalized for Pandemic (H1N1) 2009, Malaysia

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To determine effects of pandemic (H1N1) 2009 on children in the tropics, we examined characteristics of children hospitalized for this disease in Malaysia. Of 1,362 children, 51 (3.7%) died, 46 of whom were in an intensive care unit. Although disease was usually mild, ≥1 concurrent conditions were associated with higher death rates.

Transmission of pandemic (H1N1) 2009 in the Northern and Southern Hemispheres has been well documented (*1*–*12*). These reports described a relatively mild and self-limited clinical illness for most cases. However, data on disease prevalence and severity in children in the tropics are scarce. Malaysia has 132 public and 214 private hospitals. We examined the demographics, clinical presentation, and outcomes of children hospitalized for pandemic (H1N1) 2009 in 68 Ministry of Health–affiliated public hospitals in Malaysia that offered pediatric services. These 68 hospitals provide 3,757 beds for children and 101 beds in pediatric intensive care units; each year they serve ≈7,898,700 children <12 years of age. During the pandemic (H1N1) 2009 containment phase, May–July 2009, the Ministry of Health mandated that all patients with influenza-like illness (ILI) be admitted to government (public) hospitals for observation and management. During the mitigation phase from August 2009 on, only patients with moderate to severe cases of ILI were hospitalized (*13*). Influenza vaccine is used in the private sector only, and during the study period, only health care workers were vaccinated against pandemic (H1N1) 2009.

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The Study

We enrolled children <12 years of age who were hospitalized for ILI from June 18, 2009, through March 1, 2010, and for whom pandemic (H1N1) 2009 infection was confirmed by real-time reverse transcription–PCR. This study was reviewed and approved by the Malaysian Research and Ethics Committee. Informed consent was provided for patients with confirmed diagnoses.

During the study period, 1,362 children were hospitalized for ILI. The first case was diagnosed and confirmed as pandemic (H1N1) 2009 during the third week of June 2009; the number of cases peaked at week 33 and declined until week 43 of 2009 and weeks 1–9 of 2010 (Figure). The rapid decline of cases after week 33 may have resulted from a change in hospitalization criteria recommendations. From June 18 through July 2009, hospitalization rates among children <12 years, <5 years, and <2 years of age were 1.4, 1.0, and 1.1 per 100,000 children in each age group, respectively. From August 2009 through February 2010, corresponding hospitalization rates were 15.9, 23.8, and 33 per 100,000 children, respectively.

Overall median age of the hospitalized children was 3 years (interquartile range [IQR] 1–6 years); 861 (63.2%) were <5 years and 536 (39.4%) were <2 years of age. Among those who died, median age at time of death was 2 years (IQR 0–6 years). Other demographic characteristics of the cohort are shown in Table 1.

A total of 602 (44.2%) children were admitted to hospital within 48 hours of onset of clinical signs. Median interval from onset of signs to hospitalization was 3 days (IQR 1–5 days) for the overall cohort, 3 days (IQR 1–5 days) for those who survived, and 4 days (IQR 2–6 days) for those who died. Among 120 (8.8%) children whose clinical condition worsened during hospitalization, deterioration occurred within the first 24 hours after admission for 67 (55.9%). Among 657 (48.2%) patients for whom blood cultures were performed, results were positive for only 29 (4.4%). The most common pathogen isolated was *Streptococcus pneumoniae* (n = 6), followed by coagulase-negative *Staphylococcus* spp. (n = 5), *Burkholderia cepacia* (n = 4), *Klebsiella pneumoniae* (n = 2), and *Pseudomonas* spp. (n = 2). Of 6 *S. pneumoniae* isolates, 5 were documented within

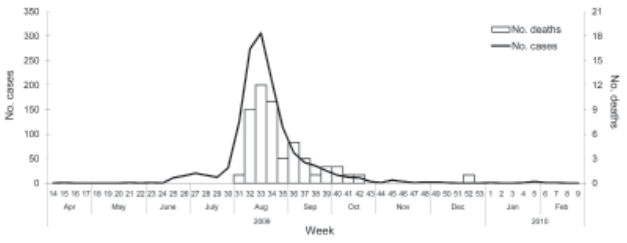

Figure. Distribution of laboratory-confirmed cases of pandemic (H1N1) 2009 and deaths in 1,362 hospitalized children, Malaysia, June 18, 2009–March 1, 2010.

48 hours of admission, including 2 from children who died. Laboratory parameters at time of admission did not differ significantly between those who survived and those who died, except for hemoglobin (odds ratio [OR] 0.71, 95% confidence interval [CI] 0.59–0.87, p = 0.001), arterial bicarbonate (OR 0.81, 95% CI 0.72–0.91, p<0.001), and serum albumin (OR 0.90, 95% CI 0.87–0.94, p<0.001). Among 1,049 children for whom chest radiographs were taken at time of admission, infiltrates were noted for 643 (61.3%), consolidation for 254 (24.2%), and pleural effusion for 4 (0.4%). No radiographic abnormalities were detected for 173 (16.5%) children. Antiviral drugs had been given to 78 (5.7%) children before admission and to 1,306 (95.6%) children after admission. The most common antiviral drug used was oseltamivir, followed by zanamivir (received by 2 [0.2%] children). An antiviral drug was given within 48 hours of onset of signs for 388 (28.5%) children, including 382 (29.1%) who survived and 6 (11.7%) who died. An antiviral drug was given >48 hours after onset of signs for 783 (57.5%) children, including 748 (57.1%) who survived and 35 (68.6%) who died. Administration of an antiviral drug within 48 hours of onset of signs was associated with a lower risk for death (OR 0.4, 95% CI 0.2–0.9; p = 0.02). Median duration of oseltamivir administration was 5 days (IQR 4–7 days). Among 1,306 children for whom data were available, 982 (75.2%) also received antibacterial drugs.

Among the same 1,306 children for whom data were available, 461 (35.3%) had a concurrent illness (Table 2); 416 (31.9%) had 1 concurrent illness and 65 (4.9%) had 2. Presence of ≥1 concurrent conditions was associated with a 4-fold increased risk for death (OR 4.4, 95% CI 2.4–8.1; p<0.001). Risk for death was higher for those with chronic lung disease (OR 2.5, 95% CI 1.1–5.6; p<0.02) than for those with other concurrent conditions. Among the 64 (4.7%) children who required inotropic support, 23 (34.3%) survived. Among all 1,362 hospitalized children, 51 (3.7%) died, including 46 (90.2%) in intensive care units and 25 (49%) who were <2 years of age (OR 1.51, 95% CI 0.86–2.64), p = 0.15). Among the 101 children who required mechanical ventilation, 49 (48.5%) died. Among the 1,352 children for whom follow-up data were available, 1,285 (95%) recovered fully and had no sequelae at time of hospital discharge, and 12 (0.9%) recovered but had sequalae (5 pulmonary, 4 neurologic, 2 renal, and 1 pulmonary and neurologic). The mortality rate for children who were <12, <5, and <2 years of age during June–July 2009 was 0.1 death per 100,000 children. Corresponding rates for August 2009–February 2010 were 0.6, 0.9, and 1.3 per 100,000 children, respectively.

Conclusions

In the tropics, pandemic (H1N1) 2009 is a relatively mild illness in children who have no concurrent condition. Serious complications such as shock and acute respiratory distress syndrome were relatively rare. However, among the small proportion for whom disease was severe, progression was rapid and death occurred within a short period. The case-fatality rate for the hospitalized cohort reported here was 3.7%, comparable to the rates of 0.1%–

Table 1. Characteristics of 1,362 hospitalized children with pandemic (H1N1) 2009, Malaysia, June 18, 2009–March 1, 2010

Characteristic	No. (%) children
Demographic	
Male sex	762 (55.9)
Age group	
0–6 mo	152 (11.2)
7–12 mo	182 (13.4)
13–23 mo	202 (14.8)
2–4 y	325 (23.9)
5–8 y	298 (21.9)
9–12 y	203 (14.8)
Ethnic group	
Malay	995 (73.1)
Chinese	109 (8.0)
Indian	83 (6.1)
Native East Malaysian*	99 (7.3)
Indigenous native	24 (1.8)
Other	52 (3.8)
Clinical sign or symptom	
Fever	1313 (96.4)
Cough	1237 (90.8)
Runny nose	794 (58.3)
Nausea	346 (25.4)
Poor feeding	310 (22.8)
Labored breathing	293 (21.5)
Diarrhea	177 (13.0)
Sore throat	164 (12.0)
Seizure	117 (8.6)
Fatigue	94 (6.9)
Headache	30 (2.2)
Abdominal pain	30 (2.2)
Altered consciousness	13 (1.0)
Vomiting	6 (0.4)
Disease severity/treatment needed	
Admission to intensive care unit	134 (9.8)
Mechanical ventilation	101 (7.4)
Supplemental oxygen†	317 (23.3)
Noninvasive ventilation‡	4 (0.3)
Complication	
Shock	57 (4.2)
Acute respiratory distress syndrome	41 (3.0)
Encephalitis/encephalopathy§	21 (1.5)
Myocarditis	8 (0.6)
Disseminated intravascular coagulation	7 (0.5)
Liver impairment	32 (2.3)
Multiple organ failure	12 (0.9)
Myoglobinuria	1 (0.07)

*Kadazan/Dusun, Melanau, Bajau, Bidayuh, Iban, Orang Ulu, Lundayeh, Kayan, Kedayan, Sabahan, Kadayan, Suluk, Tidung, Bisaya.
†Oxygen delivered by nasal cannula, nasal prong, or face mask.
‡Mechanical ventilation that does not use an artificial airway such as endotracheal tube.
§Inflammation of brain or degeneration of brain function.

Table 2. Concurrent conditions in children hospitalized for pandemic (H1N1) 2009, Malaysia, June 18, 2009–March 1, 2010*

Condition	No. (%) children			OR (95% CI)	p value
	Total	Survived	Died		
None	860(63.1)	845 (64.7)	15 (29.4)	0.2 (0.1–0.4)	<0.001
Chronic lung disease	258 (18.9)	246 (18.8)	12 (23.5)	2.5 (1.1–5.6)	0.02
Neuromuscular disease	33 (2.4)	27 (2.1)	6 (11.8)	2.5 (4.5–34.8)	<0.001
Cardiovascular disease	54 (4.0)	46 (3.5)	8 (15.7)	9.8 (3.9–24.3)	<0.001
Renal disease	18 (1.3)	16 (1.2)	2 (3.5)	–	–
Immunosuppression	18 (1.3)	15 (1.1)	3 (5.9)	–	–
Obesity	14(1.0)	13 (1.0)	1 (2.0)	–	–
Malnutrition	14 (1.0)	13 (1.0)	1 (2.0)	–	–

*OR, odds ratio; CI, confidence interval; –, numbers too small to infer from study sample.

5.1% documented by others (*4*,*9*,*14*). Hospitalization and mortality rates were proportionally higher for children <2 years of age than for children in other age groups. Severe disease leading to death was more likely for patients who had ≥1 concurrent condition. Other studies have demonstrated similar findings (*3*,*4*,*7*,*11*).

Data on concurrent conditions can help identify and prioritize patients who need prompt antiviral drug therapy and vaccination in countries with limited resources. Our finding that early administration of an antiviral drug was associated with a lower risk for death concurs with findings of other studies (*3*). Study limitations include the facts that patients with mild cases may not seek (or be brought for) medical attention and that not all cases of ILI were laboratory confirmed as pandemic (H1N1) 2009. Early initiation of antiviral therapy, especially for children with concurrent conditions, may improve outcomes.

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Human Metapneumovirus Infection in Wild Mountain Gorillas, Rwanda

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The genetic relatedness of mountain gorillas and humans has led to concerns about interspecies transmission of infectious agents. Human-to-gorilla transmission may explain human metapneumovirus in 2 wild mountain gorillas that died during a respiratory disease outbreak in Rwanda in 2009. Surveillance is needed to ensure survival of these critically endangered animals.

The world's remaining 786 mountain gorillas (*Gorilla beringei beringei*) live in 2 parks in Rwanda, Uganda, and the Democratic Republic of the Congo. An ecotourism industry for viewing human-habituated mountain gorillas in the wild is thriving in all 3 countries. Mountain gorilla tourism helps ensure the sustainability of the species by generating much-needed revenue and increasing global awareness of the precarious status of this species in the wild. Tourism, however, also poses a risk for disease transmission from humans to the gorillas.

Habitat encroachment and poaching are threats to wildlife survival, particularly in the developing world. Mountain gorillas face an additional threat from infectious diseases. Second only to trauma, infectious diseases, primarily respiratory, account for 20% of sudden deaths (*1*). The close genetic relatedness of mountain gorillas and humans has led to concerns about the potential interspecies transmission of infectious agents (*2*,*3*). Although most surveillance efforts focus on risk for humans, mountain gorillas are immunologically naive and susceptible to infection with human pathogens. The parks in which mountain gorillas live are surrounded by the densest human populations in continental Africa. In addition, research and gorilla ecotourism brings thousands of persons from the local communities and from around the world into direct and indirect contact with the gorillas. The frequency and closeness of contact is particularly pronounced in Virunga National Park, where 75% of mountain gorillas are habituated to the presence of humans.

To minimize the threat of disease transmission, the Rwandan, Ugandan, and Congolese governments restrict tourist numbers and proximity, and the Congolese wildlife authority mandates that masks be worn by persons visiting gorillas. Nonetheless, the frequency and severity of respiratory disease outbreaks among mountain gorillas in the Virunga Massif have recently increased. From May through August 2008, sequential respiratory outbreaks occurred in 4 groups of mountain gorillas accustomed to tourism in Rwanda. Between June 28 and August 6, 2009, a fifth outbreak occurred in 1 of these groups, Hirwa. We describe the Hirwa outbreak. Respiratory outbreaks were defined as more than one third of animals in a group exhibiting signs of respiratory disease (coughing, oculonasal discharge, and/or lethargy).

The Cases

The Hirwa group consisted of 12 animals: 1 adult male, 6 adult females, 3 juveniles, and 2 infants. Moderate to severe respiratory disease (≥2 characteristic signs) developed in 11 of 12 animals. Five (3 juvenile males and 2 adult females) received antimicrobial drug therapy (ceftriaxone, 50 mg/kg for adults, 100 mg/kg for infants), 4 by remote delivery and 1 while chemically immobilized. Two untreated animals (1 adult female and 1 male infant born to a symptomatic mother) died. On June 30, the adult female was first observed coughing and lethargic but still feeding. On July 3, she left her night nest in the morning but did not join her group; she exhibited severe clinical signs and was found dead on July 4 at ≈1:00 PM. The infant was 3 days old when it died on July 23. Clinical signs of respiratory illness had not been observed, although its mother showed severe clinical signs for 2–3 days before and after delivery; before delivery, she had received antimicrobial drugs by remote delivery (neither she nor her infant were handled by humans).

Gross postmortem examinations revealed bronchopneumonia in the adult and unilateral pulmonary congestion and an empty stomach in the infant. Formalin-fixed (10%) postmortem tissue samples from the adult and infant were prepared in 6 μm sections for histologic studies, stained with hematoxylin and eosin according to standard methods,

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and examined by light microscopy. Histologically, the respiratory tract of the adult was characterized by moderate mononuclear tracheitis, laryngitis, and air sacculitis; severe pulmonary alveolar histiocytosis; multifocal severe suppurative pneumonia; and multifocal pulmonary thrombosis and hemorrhage. One section of lung from the infant showed pulmonary atelectasis, congestion, mild alveolar hemorrhage, and histiocytosis. The infant also had moderate neutrophil and macrophage infiltration of the umbilicus at the body wall; neutrophilic inflammation in the media and adventitia of 1 umbilical artery at the level of the bladder; and mild, unilateral, focal, segmental, neutrophilic glomerulitis and tubulointerstitial nephritis. Coronary groove and mesenteric fat were absent.

Multiplex PCR analysis for respiratory pathogens indicated sequences of human metapneumovirus (HMPV) in serum; lung tissue; and throat, nose, anus, and vagina swabs from the adult gorilla, and in lung tissue from the infant (Table). *Streptococcus pneumoniae* was detected in lung tissue and in throat and nose swabs of the adult gorilla but not in the infant. *Klebsiella pneumoniae* was also detected in all specimens from the adult gorilla. Microbial loads were determined by quantitative PCR (Table). The sample with the highest viral load, a throat swab from the adult female (6.2×10^5 genome copies/μL), was pyrosequenced, yielding 607,484 reads comprising 3.8 kb of sequence (27.5% of the genome). Simple pairwise analysis indicated that the strain belonged to lineage B2 of HMPV (*4*). Bayesian analysis revealed close relationship of the gorilla virus to human isolates from South Africa (Figure). Sequence information for the reported HMPV has been deposited with GenBank under accession no. HM197719. Detailed methods are available in the online Technical Appendix (www.cdc.gov/EID/content/17/4/zzz-Techapp.pdf).

Conclusions

Experimental infections of cynomologus macaques with HMPV have suggested that pure infection with this virus causes minimal to mild lesions in conducting airways and increased macrophages in alveoli (*5*). However, paramyxoviruses, including HMPV, can predispose animals to bacterial pneumonia (*6*–*8*), as appeared to be the case in the adult female mountain gorilla reported here. That HMPV can be fatal for gorillas is supported by a report of a respiratory outbreak in wild, human-habituated chimpanzees in which several chimpanzees died (*2*,*9*).

We report conclusive evidence for association of a human virus with death in mountain gorillas (*2*,*3*). Viral RNA in multiple tissue samples from the adult female indicates that she was infected by an HMPV strain at the time of her death. The upper respiratory lesions were suggestive of a viral infection (*9*). The pulmonary lesions indicated a bacterial bronchopneumonia as the proximate cause of death, compatible with an etiologic agent such as *S. pneumoniae* and *K. pneumoniae*, the 2 organisms detected

Table. Results of microbiologic testing of mountain gorilla tissues, Rwanda*

Sample source and no.	Sample type	MassTag RNA panel results	HMPV viral load, genome copies/μL	MassTag DNA panel results	Bacterial load, genome copies/μL	
					Streptococcus pneumoniae	*Klebsiella pneumoniae*
Adult						
1	Serum	HMPV	2.7×10^3	*K. pneumoniae*	ND	3.2×10^7
2	Buffy coat	Negative	ND	*K. pneumoniae*	ND	4.4×10^8
3	Kidney	Negative	ND	*K. pneumoniae*	ND	6.2×10^8
4	Lung	HMPV	3.2×10^2	*S. pneumoniae*, *K. pneumoniae*	1.3×10^2	2.0×10^7
5	Heart	Negative	ND	*K. pneumoniae*	ND	1.4×10^7
6	Spleen	Negative	ND	*K. pneumoniae*	ND	9.1×10^5
7	Liver	Negative	ND	*K. pneumoniae*	ND	3.1×10^5
8	Throat swab	HMPV	6.2×10^5	*S. pneumoniae*, *K. pneumoniae*	5.5×10^4	1.4×10^5
9	Nasal swab	HMPV	2.3×10^5	*S. pneumoniae*, *K. pneumoniae*	4.3×10^4	2.4×10^6
10	Vaginal swab	HMPV	2.0×10^2	*K. pneumoniae*	ND	6.0×10^5
11	Anal swab	HMPV	4.0×10^2	*K. pneumoniae*	ND	5.5×10^2
12	Purulent discharge	Negative	ND	*K. pneumoniae*	ND	5.2×10^2
Infant						
18	Spleen	Negative	ND	Negative	ND	ND
19	Lung	HMPV	$<5.0 \times 10^1$	Negative	ND	ND
20	Liver	Negative	ND	Negative	ND	ND
21	Kidney	Negative	ND	Negative	ND	ND

*HMPV, human metapneumovirus; ND, not detected.

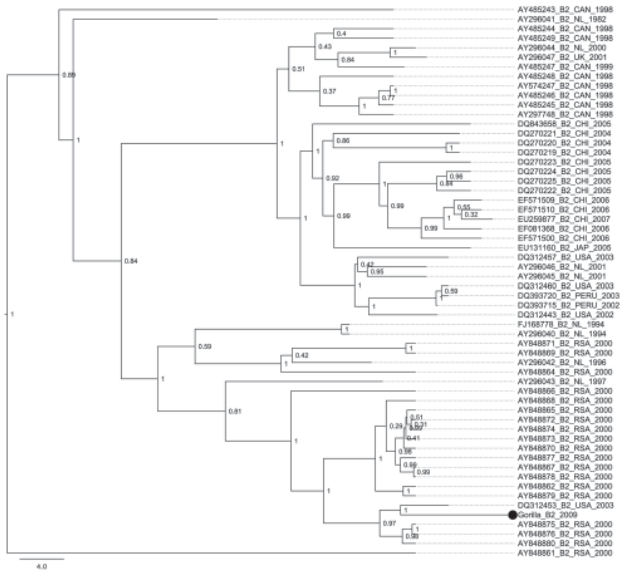

Figure. Bayesian analysis of the G gene of human metapneumovirus (HMPV) isolated from an adult female mountain gorilla that died during an outbreak of respiratory disease, Rwanda. Bayesian phylogenetic analyses of sequence differences of the HPMV glycoprotein gene were conducted by using BEAST, BEAUti, and Tracer analysis software packages (http://beast.bio.ed.ac.uk/Main_Page). Only lineage B2 HMPVs are shown. The black dot indicates the strain newly isolated from the gorilla; trees are rooted at the midpoint; scale is in years. An expanded version of this figure, showing the complete HMPV gene analysis, is available online (www.cdc.gov/EID/content/17/5/711-F.htm).

by PCR. Although the cause of death of the infant was likely inanition and acute dissemination of an umbilical infection to a kidney, detection of HMPV as the sole pathogen in the infant tissues supports the presence of this agent in the gorilla group during the respiratory disease outbreak.

The source of the virus is unknown; the strain was most recently described in South Africa. The 2 HMPV-positive animals were not handled by veterinarians or park personnel during the course of their illness. Although HMPV transmission as a result of human intervention to treat sick animals in the group is possible, it does not explain HMPV in the adult female, which died early in the outbreak before any clinical interventions were conducted. Although human proximity to mountain gorillas is essential for their conservation, also crucial is minimizing the risk for human-to–great ape transmission of respiratory pathogens.

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Highly Pathogenic Avian Influenza Virus Infection in Feral Raccoons, Japan

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Although raccoons (*Procyon lotor*) are susceptible to influenza viruses, highly pathogenic avian influenza virus (H5N1) infection in these animals has not been reported. We performed a serosurvey of apparently healthy feral raccoons in Japan and found specific antibodies to subtype H5N1 viruses. Feral raccoons may pose a risk to farms and public health.

Although all known subtypes of influenza A virus are maintained in waterfowl, these viruses have also been isolated from various avian and mammalian species. In particular, numerous reports have been made of highly pathogenic avian influenza viruses (H5N1) infecting mammals, causing lethal infections in some species (*1*,*2*). Wild mammals could transmit these viruses among other wild and domestic animals, for example, on poultry or pig farms, posing a risk for virus spread and the emergence of mutant viruses. Such viruses could have pandemic potential if they were able to infect humans, thus giving rise to a serious public health concern. Therefore, the continuous monitoring of the exposure of wild mammals to avian influenza viruses, particularly H5N1 viruses, is essential.

Raccoons (*Procyon lotor*), which belong to the *Carnivora*, are native to North America. Since the 1970s, a large number of raccoons have been imported as pets into Japan. The release and escape of these animals have resulted in a feral population widely distributed throughout Japan, which continues to increase despite an official eradication program. Recent reports, including serologic surveys and experimental infections, indicate that raccoons can be symptomatically or asymptomatically infected with low pathogenic influenza viruses, such as avian influenza subtype H4N8 or human influenza subtype H3N2 viruses, which they shed for several days, resulting in virus transmission to other raccoons by aerosol (*3*–*5*). Such findings present the possibility that wild raccoons could play a role in the transmission of subtype H5N1 viruses in a natural setting. We conducted a serologic survey for subtype H5N1 virus infection in feral raccoons in Japan.

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The Study

Raccoons are considered an invasive alien species in Japan. Recently, the growing population of feral raccoons has resulted in significant agricultural damage and prompted the initiation of eradication programs in several areas. We used a total of 1,088 serum samples collected from animals captured under this official eradication program over 3 periods in the western region of Japan and 1 period in eastern Japan during 2005–2009 for a serologic survey of avian influenza virus (H5N1) infection (Table 1). To detect antibodies specific to the H5 hemagglutinin (HA) in the serum samples, we performed a virus neutralization (VN) test (*6*) with 2 subtype H5N1 viruses, A/Indonesia/3006/2005 (clade 2.1.3) and A/whooper swan/Mongolia/4/2005 (clade 2.2). As an initial screening step, we used the serum specimens (1:5 dilution) after receptor-destroying enzyme treatment of the serum to remove nonspecific inhibitors. The VN antibody-positive serum samples were then further tested for their reactivity by using a panel of influenza viruses of multiple subtypes (Table 2) as well as Western blot analysis (Figure 1). In these assays, we found a total of 10 serum specimens that were positive for VN antibody to subtype H5N1 viruses, representing 0.9% positivity. The A-1 to A-6 serum specimens, which were collected from animals captured within a 10 km^2 area, strongly reacted to A/whooper swan/Mongolia/4/2005 (clade 2.2) and more weakly to other clades of subtype H5N1, H5N2, and H5N3 viruses. These serum specimens did not react to viruses of other HA subtypes, including H1, H3, H7, and H9. Of note, the A-2, A-3, and A-4 animals were from the same litter captured at a lair, which suggests that the detected VN antibodies in these samples might be maternal antibodies from their uncaptured mother, who may have been infected with a subtype H5N1 virus. It is possible that 2 viruses of clade 2.2, which had slightly different antigenicities, may have infected raccoons in this area, as indicated by the different patterns of cross-reactive VN titers to subtype H5N1 clade 1 and H5N3 viruses. One group consisted of A-1 to A-4 and the other of A-5 and A-6. The B-1 and B-2 samples from animals captured at a 25-km distance strongly reacted to both subtype H5N1

Table 1. Summary of serologic test results showing avian influenza (H5N1) antibody–positive samples from feral raccoons, Japan*

Region/period	No. positive/no. samples (%)	ID no., positive samples	Date raccoon captured	Animal body mass, kg	Animal sex
Western Japan					
2005 May–2006 Dec	6/221 (2.7)				
		A-1	2006 Apr 30	5.6	F
		A-2	2006 May 2	0.3	M
		A-3	2006 May 2	0.3	M
		A-4	2006 May 2	0.3	M
		A-5	2006 May 17	10.9	M
		A-6	2006 May 25	9.1	M
2007 Jun–2008 May	2/84 (2.4)				
		B-1	2007 Jun 28	5.3	M
		B-2	2008 Jan 20	7.2	M
2009 Apr–2009 Sep	0/110				
Eastern Japan					
2007 May–2008 Oct	2/683 (0.3)				
		C-1	2008 May 8	4.0	F
		C-2	2008 Jul 1	7.2	M
Total	10/1,088 (0.9)				

*ID, identification.

clades 2.2 and 2.5 viruses. Given that the subtype H5N1 clade 2.5 virus has not circulated since 2004 and that the clade 2.2 virus was more highly reactive than the clade 2.5 virus, these raccoons were likely infected with clade 2.2 viruses, as supported by timing with poultry outbreaks. By contrast, the C-1 and C-2 samples from raccoons captured in eastern Japan reacted strongly to A/whooper swan/Akita/1/2008 (clade 2.3.2), unlike the samples from western Japan, indicating that the C-1 and C-2 animals were infected with a virus of this clade. Together, these data suggest that feral raccoons in Japan have been infected with subtype H5N1 viruses of different clades.

To assess the presence of anti-neuraminidase (NA) antibodies in the serum samples, we used an NA-inhibition (NI) assay for the VN-positive samples and found marked inhibition of the NA activity of the N1 subtype (Figure 2). We also performed the standard NI assay using another N1 virus, A/swine/Iowa/15/30 (H1N1), to avoid nonspecific NA inhibition by H5 antibodies, for 2 VN-positive serum specimens (A-6 and C-2) and found that A-6 and C-2 had positive NI titers of 20 and 80, respectively. These data demonstrate that VN-positive raccoon serum specimens contain anti-H5N1 antibodies, indicating that raccoons have been infected with subtype H5N1 viruses.

Conclusions

Japan has experienced 3 outbreaks of highly pathogenic subtype H5N1 viruses. In the first in early 2004, clade 2.5 subtype H5N1 viruses were detected in poultry farms in western Japan. The second, in early 2007, involved the isolation of clade 2.2 subtype H5N1 viruses from poultry in western Japan. The third occurred in mid-2008, when clade 2.3.2 viruses were isolated from diseased swans in the lakes in the northern area of eastern Japan. All of these outbreaks were contained by prompt culling of birds. Since 2008, subtype H5N1 viruses have not been reported in any poultry or wild migratory birds under the government surveillance program. Our data indicate that raccoons

Table 2. Cross-reactivity of avian influenza (H5N1) virus neutralizing antibody–positive samples from feral raccoons, Japan*

ID no., positive samples	Virus antigen subtype									
	H1N1, H3N2, H7N6, H7N7, H9N2	H5N1 clade							H5N2	H5N3
		1	2.1.3	2.2a	2.2b	2.3.2	2.3.4	2.5		
A-1	<8	<8	8	32	8	<8	<8	16	<8	<8
A-2, 3, 4	<8	<8	64	256	64	<8	<8	128	<8	<8
A-5	<8	32	64	512	64	8	8	256	<8	64
A-6	<8	32	64	1,024	128	8	8	128	8	16
B-1	<8	32	32	256	128	8	16	256	8	64
B-2	<8	8	16	256	64	<8	8	256	8	64
C-1	<8	8	16	256	16	1,024	<8	64	<8	<8
C-2	<8	<8	16	512	16	256	<8	256	<8	<8

*We used A/PR8/34 (H1N1), A/Aichi/2/68 (H3N2), A/quail/Aichi/1/09 (H7N6), A/seal/MA/1/80 (H7N7), A/Hong Kong /1073/99 (H9N2), A/Vietnam/1194/04 (H5N1; clade 1), A/Indonesia/3006/05 (H5N1; clade 2.1.3), A/whooper swan/Mongolia/4/05 (H5N1; clade 2.2a), A/chicken/Miyazaki/K11/07 (H5N1; clade 2.2b), A/whooper swan/Akita/1/08 (H5N2; clade 2.3.2), A/Hanoi/30850/05 (H5N1; clade 2.3.4), A/chicken/Yamaguchi/8/04 (H5N1; clade 2.5), A/chicken/Ibaraki/1/05 (H5N2), and A/whooper swan/Shimane/499/83 (H5N3) as antigens for the virus neutralization test. Representative data from 3 independent experiments are shown.

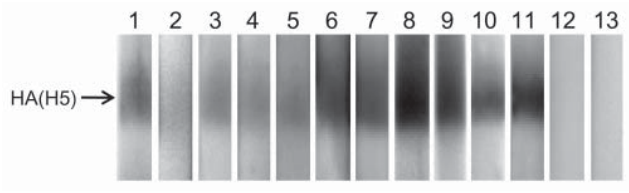

Figure 1. Western blot analysis of virus neutralization (VN)–positive raccoon serum specimens. A/whooper swan/Mongolia/4/05 (H5N1) virus (clade 2.2) was purified through a 25% sucrose cushion and used as an antigen under nonreducing conditions in the Western blot assay. After blocking with 5% skim milk, each raccoon serum specimen (1:100 dilution) was incubated for 1 h and then reacted with horseradish peroxidase (HRP)–labeled protein A/G (Pierce Chemical Co., Rockford, IL, USA) and subjected to chemiluminescence detection (ECL Plus, GE Healthcare UK, Ltd, Chalfont St. Giles, UK). Serum from a mouse infected with A/whooper swan/Mongolia/4/05 was used as a marker. The negative control reaction when only HRP-protein A/G is used is also shown. HA, hemagglutinin; Ab, antibody. Lane 1, anti-H5N1 polyclonal Ab; lanes 2–11, VN-positive serum (2, A-1; 3, A-2; 4, A-3; 5, A-4; 6, A-5; 7, A-6; 8, B-1; 9, B-2; 10, C-1; 11, C-2); lane 12, VN-negative serum; lane 13, negative control.

in western Japan were likely infected with the clade 2.2 viruses, whereas those in eastern Japan were infected with the clade 2.3.2 virus. Notably, some antibody-positive raccoons in western Japan were captured 6 months before the poultry outbreak with clade 2.2 virus, suggesting that a clade 2.2 subtype H5N1 virus had invaded Japan by 2006.

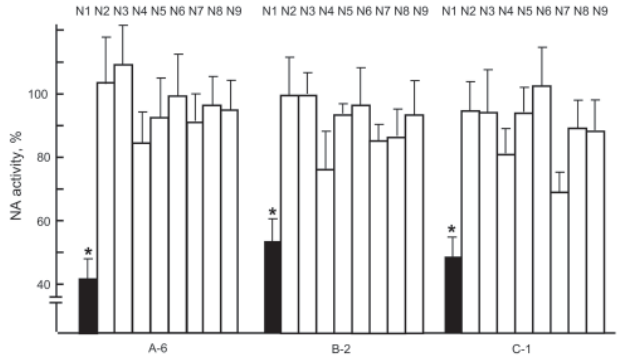

Figure 2. Neuraminidase (NA) inhibition by virus neutralization (VN)–positive raccoon serum samples. Each of the N1 to N9 viruses, consisting of A/whooper swan/Mongolia/4/05 (H5N1), A/Hong Kong/1073/99 (H9N2), A/whooper swan/Shimane/499/83 (H5N3), A/turkey/Ontario/6188/68 (H8N4), A/duck/Alberta/60/76 (H12N5), A/duck/England/56 (H11N6), A/seal/Massachusetts/1/80 (H7N7), A/duck/Ukraine/1/63 (H3N8), and A/duck/Memphis/546/74 (H11N9), was incubated with a VN-positive serum sample (A-6, B-2, or C-1) for 1 h at 37°C, and viral NA activity was then measured (*6*). Data are shown as percentage of activities compared with incubation with VN-negative serum samples (100%). Three independent tests were performed, and significant reduction of NA activity ($p<0.05$, *t* test with 2-tailed analysis) was observed only for N1 virus (*). HA, hemagglutinin. Error bars indicate SDs of 3 independent tests.

We cannot determine by seropositive text results the exact date when the viruses infected the raccoons, because the duration of naturally acquired antibody to subtype H5N1 virus in this species is unknown. Recent data indicate that this animal maintains a detectable serum antibody response for at least 9 months after natural exposure to influenza viruses of other HA subtypes such as H1, H3, and H4 (*7*). In humans, a detectable antibody response to seasonal viruses can last >5 years (*8*) and in swine antibodies to the virus have been detected 28 months postinfection (*9*).

Because wild raccoons are omnivores and highly opportunistic at exploiting foods they prefer, whenever available they could eat diseased or dead migratory birds from areas where subtype H5N1 viruses are enzootic. They also sometimes attack poultry farms for food, creating the potential to transmit virus to domestic poultry. In addition, the increasing likelihood for contact between wild raccoons and humans elevates the possibility of human infection with these viruses, posing risks to public health and increasing the possibility of the emergence of mammalian-adapted mutant viruses with pandemic potential. Further investigation and surveillance of influenza virus infections in peridomestic animal species are needed to better understand influenza ecology.

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Secondary and Tertiary Transmission of Vaccinia Virus from US Military Service Member

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During February and March 2010, the New York State Department of Health investigated secondary and tertiary vaccinia contact transmission from a military vaccinee to 4 close contacts. Identification of these cases underscores the need for strict adherence to postvaccination infection control guidance to avoid transmission of the live virus.

Vaccinia virus (VACV) is the live viral component of smallpox vaccine. Exposure to the vaccination site can result in contact transmission or inadvertent autoinoculation, which often is self-limited (*1*,*2*). However, severe complications can occur, especially in persons with underlying risk factors (e.g., immunodeficiencies, atopic dermatitis, or pregnancy) (*1*). During December 2002–May 2009, the reported rate of contact transmission for US military personnel was 5 cases per 100,000 persons; intimate and sports-related contact were the most commonly cited risks (*3*).

On March 11, 2010, the New York State Department of Health (NYSDOH) was notified of a suspected case of vaccinia in a person who had been exposed to a military service member recently vaccinated against smallpox. NYSDOH and the Centers for Disease Control and Prevention (CDC) conducted an investigation to identify the source of infection and potential contacts. One additional case of contact transmission from the primary vaccinee and 2 cases of tertiary transmission were confirmed. This investigation underscores the need for strict adherence to postvaccination infection control guidance to avoid transmission of the live virus.

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The Study

On February 23, 2010, a military service member preparing for deployment received smallpox vaccination and was counseled by the US Department of Defense about postvaccination care and infection control. On February 27, the index patient wrestled 2 persons in a semiprofessional match, during which the dressing covering the vaccination site was detached. Within 3 days, skin lesions developed in both contacts. One of these 2 wrestlers participated in another wrestling match on March 5, exposing a third person in whom lesions on the chest developed. A fourth contact, a household member of a wrestler from the February 27 match, had lesions develop on the face. Test results for all 4 persons were positive for VACV (Table). Each case was followed through resolution of lesions. Skin lesions developed in 3 additional contacts. All were examined, and test results of specimens were negative for VACV (Figure 1).

Case-Patient 1

On March 11, a 26-year-old male wrestler with no noteworthy medical history visited a dermatologist after being referred by his private physician who had prescribed trimethoprim/sulfamethoxazole for presumed methicillin-resistant *Staphylococcus aureus* from lesions on his face, neck, and chest (Figure 2). The lesions developed suddenly, starting March 1, two days after wrestling. Molluscum contagiosum and impetigo were included in the differential diagnosis, and a culture was sent to a local laboratory to test for methicillin-resistant *S. aureus*. The dermatologist notified the local health department of the patient's possible exposure to VACV. A NYSDOH public health physician evaluated the patient on March 12. On examination, he had clinically compatible VACV lesions, including papular, umbilicated lesions with overlying vesicles and a few pustules.

Case-Patient 2

On March 11, a 24-year-old male wrestler with no noteworthy medical history was contacted by the NYSDOH because he was a wrestling contact of the vaccinee. He reported lesions on his face, neck, chin, and left eye that developed several days after the wrestling event and were associated with substantial pruritus, exudate, and erythema. At his public health evaluation on March 12, he had numerous papular lesions that were umbilicated with overlying vesicles, several of which were draining serous fluid, and VACV was clinically diagnosed. He

Table. Cases of laboratory-confirmed secondary and tertiary transmission of vaccinia virus from US military service member, New York, USA, 2010*

No.†	Transmission type	Age, y/sex	Exposure			Illness		Underlying risk factors	VIG	Duration of lesions, wk
			Date	Source	Type	Onset	Location of lesions			
1	Secondary	26/M	Feb 27	Military vaccinee	Wrestling	Mar 1	Face, neck, chest, arm	None	No	≈3
2	Secondary	24/M	Feb 27	Military vaccinee	Wrestling	≈Mar 1	Face, neck, chin, eye	None	No‡	≈3
3	Tertiary	25/M	Mar 5	Case-patient 1	Wrestling	Mar 7	Chest, trunk, arm	Mild eczema	No	≈7
4	Tertiary	29/F	After March	Case-patient 1	Household	Mar 9	Face, mandible, nostril	None	Yes	≈3

*VIG, varicella immune globulin.
†Case-patient no.
‡Use of VIG not indicated; treated with trifluridine ophthalmic solution (*3*).

had involvement of the left lower eyelid with substantial erythema and early blepharitis. He received slit-lamp examination by a local ophthalmologist, who after consultation with CDC and NYSDOH, treated him with trifluridine ophthalmic solution (*4*). The blepharitis and eyelid erythema resolved within 48 hours after initiation of trifluridine.

Case-Patient 3

On March 10, a 25-year-old male wrestler and roommate of the vaccinee sought treatment for vesicular lesions on his trunk and chest. His medical history included recent mild eczema involving his hands. He reported that several pruritic papules had developed 2 days after wrestling case-patient 1. The physician thought the lesions appeared to be molluscum contagiosum, but given the patient's history, consulted an infectious disease physician, who observed lesions compatible with VACV. At the public health clinical evaluation on March 12, the patient had grouping of vesicular lesions with central umbilication on mildly erythematous bases on his trunk (Figure 2), with a solitary lesion near his left areola and on the volar aspect of his right forearm. VACV was clinically diagnosed.

Case-Patient 4

On March 9, lesions developed along the mandible of the 29-year-old household contact of case-patient 1. On March 11, she was evaluated by a dermatologist, who performed a punch biopsy for suspected herpes. Over the next several days, fever, chills, arthralgias, and submandibular swelling developed. She was seen by the NYSDOH public health physician on March 16, and VACV was clinically diagnosed. The most plausible route of exposure was a shared hand towel with case-patient 1. Over the next several days, her lesions started draining and became substantially more erythematous and painful. She reported a lesion in her right nostril on March 18. After consultation between her physician, NYSDOH, and CDC, varicella immune globulin was released from the Strategic National Stockpile for administration because of the new mucosal involvement (*5*). Varicella immune globulin was administered with no complications on

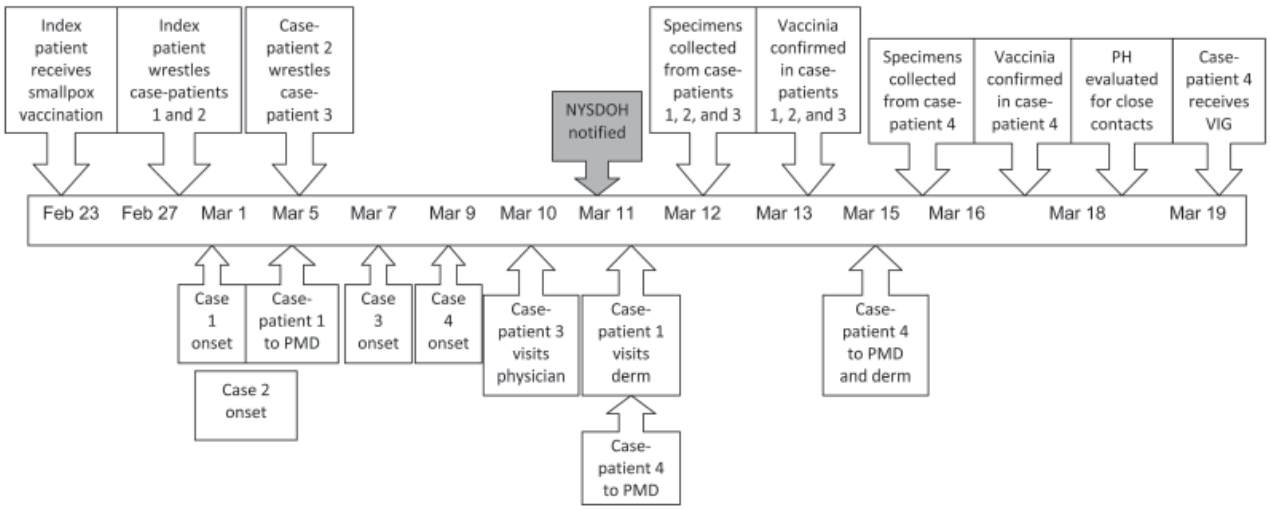

Figure 1. Timeline of investigation of secondary and tertiary transmission of vaccinia virus from a US military service member, New York, USA, 2010. NYSDOH, New York State Department of Health; PH, public health; VIG, varicella immune globulin; PMD, private physician; derm, dermatologist.

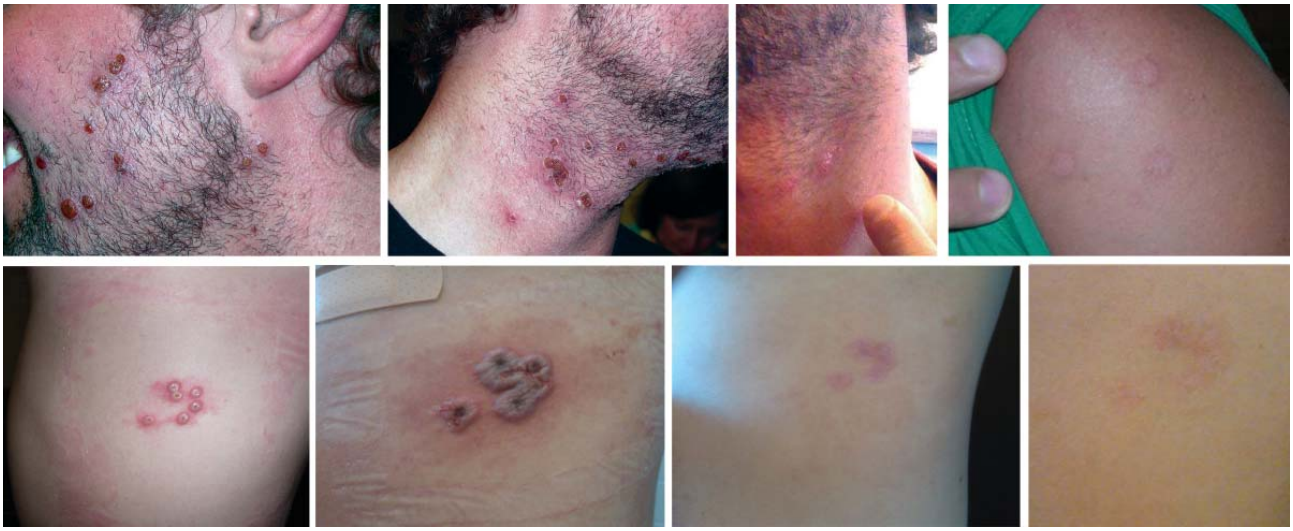

Figure 2. Vaccinia lesions in patients with secondary and tertiary cases, New York, USA, 2010. Top row, case-patient 1; bottow row, case-patient 3.

March 19 at a local hospital. The patient reported that the pain associated with her lesions resolved over 24 hours. Her nostril lesion resolved on March 22 with the loss of a small scab.

All 4 cases were clinically diagnosed vaccinia. Samples were obtained by unroofing vesicles, collecting the tissue, performing slide touch preps of the unearthed base of each vesicle, and obtaining viral swabs by using pox collection kits that had been distributed through NYSDOH. All patient specimens were tested at the NYSDOH Wadsworth Center (Albany, NY, USA) by real-time PCR, and preliminary results indicated VACV.

VACV subsequently was confirmed in all 4 patients by real-time PCR at the NYSDOH Wadsworth Center. The complete hemagglutinin gene was sequenced by CDC for 3 of the samples (cases 1, 3, 4) and was identical to that of ACAM2000 (*6*).

NYSDOH provided appropriate transmission precautions and wound care instructions to all 4 case-patients (*7*). Follow-up continued until lesion resolution; no additional VACV cases were identified. Vaccine Adverse Event Reporting System reports were submitted for each case.

Conclusions

In 2003, members of the military, selected health care workers, public health personnel, and first responders began receiving smallpox vaccinations as part of bioterrorism preparedness (*8*). Although smallpox vaccination campaigns directed toward health care workers and public health officials ended in January 2008 (*9*), smallpox vaccinations continue for military service members. This case report illustrates the need to ensure that military vaccinees understand the risk associated with contact transmission. Regions with active military smallpox vaccination programs need to maintain awareness among community medical providers, health departments, and laboratories to facilitate recognition, correct diagnosis, and appropriate response to inadvertent inoculation of vaccinia virus to help limit further transmission. Especially in areas with ongoing smallpox vaccination programs, appropriate materials for the collection of specimens need to be maintained. Finally, updates are needed on identification of VACV cases, along with the notification and involvement of public health.

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High Rates of *Staphylococcus aureus* USA400 Infection, Northern Canada

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Surveillance of *Staphylococcus aureus* infections in 3 northern remote communities of Saskatchewan was undertaken. Rates of methicillin-resistant infections were extremely high (146–482/10,000 population), and most (98.2%) were caused by USA400 strains. Although USA400 prevalence has diminished in the United States, this strain is continuing to predominate throughout many northern communities in Canada.

Over the past decade, community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections have rapidly emerged in Canada (*1*). These CA-MRSA strains are causing infections in often young otherwise healthy persons with no traditional health care–associated risk factors (*2*), linked with increased illness severity and deaths (*3*), and now entering and being disseminated within health care facilities (*4*). In comparison to the incidence of CA-MRSA infections in large urban centers across Canada, which has been addressed through the ongoing efforts of the Canadian Nosocomial Infection Surveillance Program (*1*), little attention has been directed at the emerging problem of CA-MRSA or CA-methicillin-susceptible *S. aureus* (MSSA) in rural and northern communities of Canada. In this study, active surveillance was undertaken in 3 remote northern communities to assess the prevalence and effects of MRSA and MSSA infections.

The Study

Clinically significant MRSA and MSSA isolates, identified during January 2006–March 2008, within 3 select communities (sites A–C) in northern Saskatchewan were included in this surveillance study. Site B also included 1 adjoining community, and sites A and B also included additional First Nations Reserves serviced by the community. Each site faced significant socioeconomic challenges. A total of 1,280 isolates, obtained from skin and soft tissue infections (SSTIs), urinary tract infections, upper respiratory tract infections, and lower respiratory tract infections, were identified as *S. aureus*. A high proportion of these isolates, 692 (54.1%) of 1,280, were MRSA. Over the 2-year study period, rates of MRSA and MSSA infections in the 3 communities ranged from 146–482/10,000 and 112–329/10,000 population, respectively. Trends of seasonality were apparent for MRSA infections, with the highest rates being observed in the third and fourth quarters of the year (Figure 1). Overall, the highest quarterly rates of MRSA and MSSA infections were observed at site C, with 738/10,000 and 610/10,000 population, respectively.

The highest proportion of MRSA (30.4%) and MSSA (32.1%) infections were identified in children <10 years of age (Figure 2). Compared to MSSA infections, MRSA infections were statistically more likely to be causing infections in persons <30 years of age (odds ratio [OR] 1.46, 95% confidence interval [CI] 1.14–1.86, p = 0.002) and less likely to be causing infections in patients >60 years of age (OR 0.33, 95% CI 0.20–0.567), p<0.001) (Figure 2). No significant difference was found in gender between those who acquired MRSA (46.7% male) and MSSA (53.3% female, 49.4% male) infections.

Most MRSA (98.6%) and MSSA (91%) isolates were obtained from SSTIs. Further analysis of SSTIs, comparing where on the body the infections were seen, showed significantly more MRSA infections in the axillae (OR 3.04, 95% CI 1.39–6.89, p = 0.004), buttocks (OR 2.1, 95% CI 1.27–3.49, p = 0.003), and trunk (OR 2.25, 95% CI 1.54–3.31, p = <0.001) than MSSA infections. MRSA infections were significantly less likely to be found in feet (OR 0.29, 95% CI 0.18–0.45, p<0.001), hands (OR 0.45, 95% CI 0.3–0.68, p<0.001), and face or head (OR 0.66, 95% CI 0.48–0.90, p = 0.009). Of the additional infection sites included in this study, MSSA infections were statistically more likely to be identified in lower respiratory tract infections (OR 5.6, 95% CI 1.5–24.62, p<0.05) and urinary tract infections (OR 6.76, 95% CI 2.87–16.71, p<0.001).

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[1]Members of the Northern Antibiotic Resistance Partnership are listed at the end of this article.

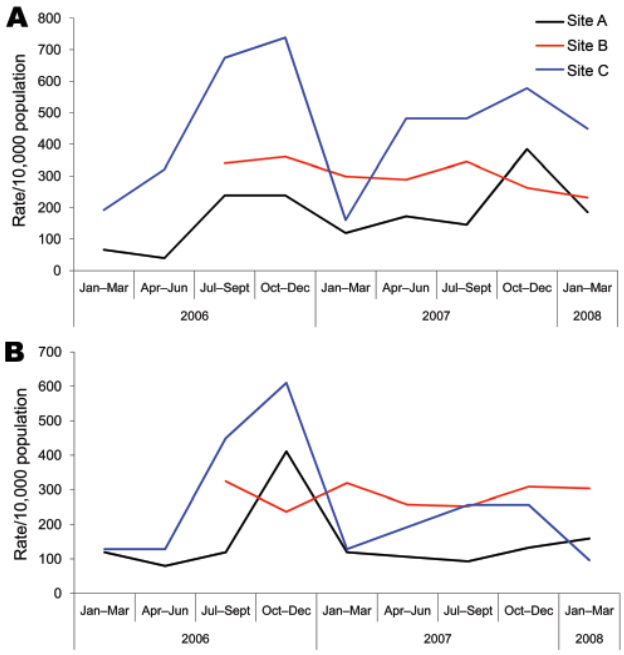

Figure 1. Crude rates of community-acquired methicillin-resistant *Staphylococcus aureus* (A) and methicillin-susceptible *S. aureus* (B) infections per 10,000 population in 3 select communities (sites A, B, and C) of northern Saskatchewan, Canada.

A subset of 665 isolates were further characterized by antimicrobial drug susceptibility testing (Table 1). In comparison to MSSA, MRSA were significantly more likely to be susceptible to clindamycin, erythromycin, fusidic acid, and gentamicin, but were more likely to be resistant to mupirocin (Table 1). In regards to the clindamycin-resistant isolates, 3 (18.8%) of the 16 MRSA isolates and 73 (93.6%) of the 78 MSSA isolates were inducible. For mupirocin-resistant isolates, all 328 of the MRSA isolates, but only 54 (70.1%) of the 77 MSSA isolates, displayed high level resistance (≥128 μg/mL).

Pulsed-field gel electrophoresis (PFGE) showed that most MRSA isolates (372/379, 98.2%) were USA400. The remaining 7 MRSA isolates were identified as CMRSA10 (USA300, sequence type (ST) 8) (n = 5), CMRSA2 (USA100/800, ST5) (n = 1), and CMRSA8 (EMRSA15, ST22) (n = 1). As anticipated, PFGE revealed much greater genetic diversity among the MSSA strains circulating in these regions than in MRSA strains. Notably, however, most MSSA PFGE fingerprints (79.2%) were related to highly successful Canadian epidemic MRSA strains, a finding that was further confirmed by using *spa* typing (*5*) (Table 2).

MRSA isolates were more likely to harbor the genes encoding Panton-Valentine leukocidin than were MSSA isolates, 95.5% versus 5.2%, respectively. The PFGE and *spa* types of the 15 Panton-Valentine leukocidin–positive MSSA isolates were associated with the CA-MRSA epidemic strain types USA400, USA300, and USA1000 (Table 2).

Conclusions

Rates of MSSA and MRSA infections in these 3 northern Saskatchewan communities (112–482 cases/10,000 population) far exceed MRSA rates reported in the neighboring provinces of Manitoba ≈16/10,000 population) (*6*) and Alberta (10.7/10,000 population) (*7*), as well as benchmark hospital rates provided by the Canadian Nosocomial Infection Surveillance Program (3.43 cases/10,000 patient days) (*1*). The high rates of *S. aureus* infections in remote northern Saskatchewan communities has been attributed to a combination of risk factors, including overcrowding and poor housing conditions, inadequate hygiene, preexisting skin conditions, and previous high usage of antimicrobial drugs (*8*).

USA400 was by far the predominant strain type in all 3 communities, accounting for >98% of the MRSA isolates. USA400 was first reported in Manitoba as an outbreak in the southern region in the late 1990s, but has since spread to the northern regions of the province from 2000 to 2004 (*9*). USA400 was thereafter seen in a central eastern Saskatchewan community adjacent to the Manitoba border (*2*) and has since disseminated as far north as Nunavut (*10*) and southwestern Alaska (*11*).

Because MRSA and MSSA SSTIs tended to be identified more frequently from different body sites, it is appealing to speculate that CA-MRSA strains, such as USA400, might also colonize different body sites (e.g., axillae or intestines) more efficiently than other strains of *S. aureus*. This hypothesis coincides with a recent report in which nasal colonization was less likely in patients with CA-MRSA SSTIs than in those with hospital-acquired

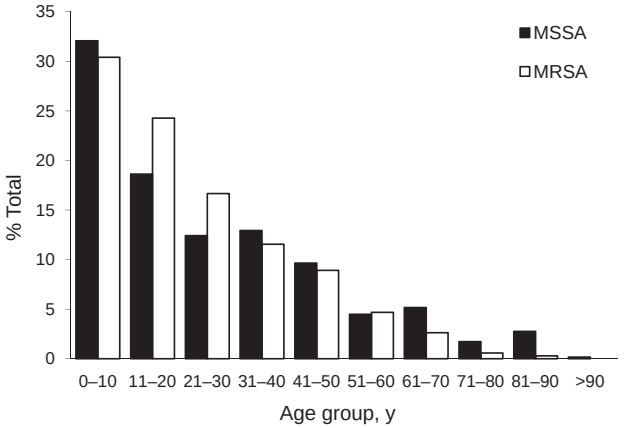

Figure 2. Age distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) infections in 3 select communities of northern Saskatchewan, Canada.

Table 1. Broth microdilution antimicrobial susceptibilities of select MRSA and MSSA isolates, northern Canada, 2006–2008*

Antimicrobial drug	MRSA isolates, n = 379				MSSA isolates, n = 286					
	% R	MIC range	MIC_{50}	MIC_{90}	% R	MIC range	MIC_{50}	MIC_{90}	p value	OR (95% CI)
Clindamycin	4.2	≤0.25–>8	≤0.25	≤0.25	27.3	≤0.25–>8	≤0.25	≤0.25	<0.001	0.12 (0.06–0.21)
Erythromycin	5.5	≤0.25–>8	1	2	28.0	0.5–>8	2	>8	<0.001	0.15 (0.09–0.26)
Vancomycin	0	≤0.25–2	1	1	0	0.5–2	1	1	NS	–
SXT	0	≤0.25–2	≤0.25	≤0.25	0	≤0.25–2	≤0.25	≤0.25	NS	–
Tetracycline	0.3	≤2–>16	≤2	≤2	0	≤2	≤2	≤2	NS	–
Ciprofloxacin	2.4	≤0.12–>8	0.5	0.5	1.7	0.25–>8	0.5	0.5	NS	–
Rifampin	0	≤0.25	≤0.25	≤0.25	0	≤0.25	≤0.25	≤0.25	NS	–
Fusidic acid	2.1	≤0.06–>8	0.12	0.25	7.7	≤0.06–>8	0.25	0.5	0.001	0.26 (0.1–0.62)
Linezolid	0	0.5–4	2	4	0	1–4	4	4	NS	–
Gentamicin	1.6	≤0.5–>16	≤0.5	≤0.5	8.0	≤0.5–>16	≤0.5	1	<0.001	0.18 (0.07–0.48)
Mupirocin	86.5	≤0.12–>128	>128	>128	26.9	≤0.12–>128	0.5	>128	<0.001	17.46 (11.56–26.43)
Synercid	0	≤0.25–0.5	≤0.25	0.5	0	≤0.25–1	0.5	0.5	NS	–
Nitrofurantoin	0	≤32	≤32	≤32	0	≤32	≤32	≤32	NS	–

*Values are mg/mL except as indicated. MRSA, methicilllin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; R, resistant; OR, odds ratio; CI, confidence interval; MIC_{50}, 50% minimum inhibitory concentration; MIC_{90}, 90% inhibitory concentration; SXT, sulfamethoxazole/trimethoprim; NS, not significant.

MRSA SSTIs (*12*). Intestinal carriage of *S. aureus* has been implicated as a risk factor for infection (*13*) and could be a strong contributor to environmental dissemination and transmission (*14*). This possibility was recently further supported by the results of a study in which the rectal carriage, but not nasal carriage, of USA300 was strongly associated with SSTIs in children (*15*). Further study is required to determine whether specific lineages of *S. aureus* are more proficient colonizers at non-nasal colonization sites, what host/bacteria genetic factors are involved, and whether this colonization plays a role in the high success of these CA-MRSA strain types.

To address the high rates of *S. aureus* infections in northern Saskatchewan, physician treatment algorithms and educational materials have been provided throughout many northern communities and schools in Saskatchewan. These materials are all freely available (www.narp.ca) and are intended to promote proper antimicrobial drug usage and hygiene to diminish the spread of *S. aureus* disease.

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Table 2. Relationship of molecularly characterized MSSA isolates to MRSA epidemic strain types*

MRSA PFGE epidemic types (MLST)	No. (%) related MSSA isolates	PVL positive	Predominant *spa* type†
CMRSA1/USA600 (ST45)	38 (13.3)	0	t065 (n = 23)
CMRSA2/USA100/800 (ST5)	77 (26.9)	0	t311 (n = 46)
CMRSA4/USA200 (ST36)	30 (10.5)	0	t012 (n = 12)
CMRSA7/USA400 (ST1)	12 (4.2)	12	t128 (n = 8)
CMRSA10/USA300 (ST8)	3 (1.1)	2	t008 (n = 2)
USA700 (ST72)	1(0.4)	0	t148 (n = 1)
ST97	18 (6.3)	0	t2728 (n = 11)
USA1000 (ST59)	33 (11.5)	1	t163 (n = 27)
USA1100 (ST30)	1 (0.4)	0	t122 (n = 1)

*n = 286 MSSA isolates. MSSA, methicilin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; PVL, Panton-Valentine leukocidin; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST, sequence type.
†www.ridom.de.

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Animal Movement and Establishment of Vaccinia Virus Cantagalo Strain in Amazon Biome, Brazil

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To understand the emergence of vaccinia virus Cantagalo strain in the Amazon biome of Brazil, during 2008–2010 we conducted a molecular and epidemiologic survey of poxvirus outbreaks. Data indicate that animal movement was the major cause of virus dissemination within Rondônia State, leading to the establishment and spread of this pathogen.

Outbreaks of vaccinia virus (VACV) infection in dairy cows and dairy workers have been frequently reported in Brazil during the past decade, mainly within the southeastern region (*1*–*6*), except for central Goiás (*2*) and Tocantins State in the northeastern boundary of the Brazilian Amazon biome (Figure 1) (*7*). VACV Cantagalo strain (CTGV) was first detected in 1999 and associated with several outbreaks (*1*,*5*,*7*). Related strains have been reported in subsequent outbreaks (*2*–*4*,*6*).

Mato Grosso State is partially inserted into the southeastern Amazon biome (Figure 1) and has the largest cattle population of Brazil, ≈26 million (*8*). It is the main connection with the Amazon states, especially with Rondônia. Rondônia has nearly 11.2 million cattle and is the largest milk producer in the region, yielding >720 million liters/year (*8*). Therefore, intense livestock trade occurs through the so-called Amazon Gate of Mato Grosso and Rondônia. Both states had previously been considered poxvirus disease–free.

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The Study

From June 2008 through June 2010, we investigated outbreaks of poxvirus-related disease on 56 dairy farms of Mato Grosso and Rondônia (Figure 1). A probable case was defined as illness observed in any cattle from these farms that showed clinical signs consistent with a poxvirus-

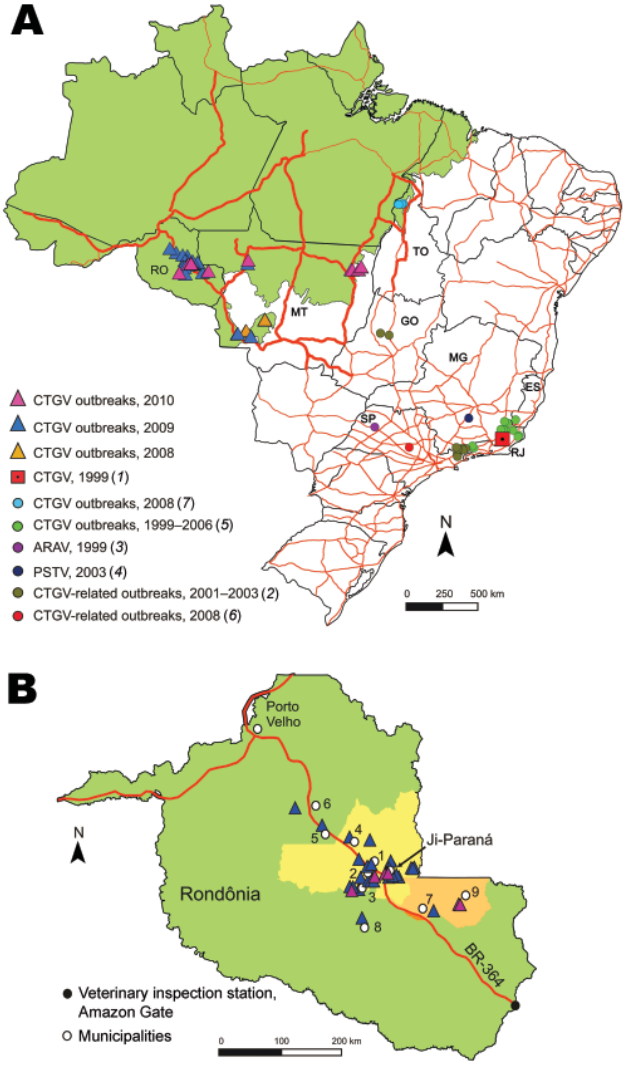

Figure 1. Location of vaccinia virus Cantagalo strain (CTGV)–related outbreaks. A) Brazilian states where CTGV-related outbreaks have been reported. RJ, Rio de Janeiro; SP, São Paulo; ES, Espírito Santo; MG, Minas Gerais; GO, Goiás; TO, Tocantins; MT, Mato Grosso; RO, Rondônia. B) An enlarged map of Rondônia showing the location of the outbreaks along highway BR-364. The 2 largest dairy regions and the municipalities referred in this article are also shown: 1, Ouro Preto D'Oeste; 2, Teixeirópolis; 3, Urupá; 4, Jaru; 5, Cacaulândia; 6, Ariquemes; 7, Cacoal; 8, Nova Brasilândia D'Oeste; 9, Espigão D'Oeste. Green shading, Amazon biome; light yellow shading, main dairy region; light orange shading, second-largest dairy region. Thin red lines indicate the Brazilian highway network and thick red lines the main highways of the Amazon region. Maps were constructed by using ArcGIS version 9.3.1 (www.esri.com) based on the location coordinates. ARAV, vaccinia virus Araçatuba strain; PSTV, vaccinia virus Passatempo strain.

Table 1. Results of PCR analysis and investigation of sick animals during outbreaks of vaccinia virus Cantagalo strain infection in the Amazon biome, Brazil, 2008–2010

Farm locations	No. farms tested/total no. farms*	No. sick animals/total no. animals							
		Age ≤12 mo		Age 13–23 mo		Age ≥24 mo			
		F	M	F	M	F	M	Total	Cohort†
Mato Grosso	12/21	26/535	28/491	0/579	0/412	277/1,611	0/510	331/4,138	331/2,637 (12.55)
Rondônia	11/35	117/1,287	95/1,823	0/1006	0/892	688/4,660	0/893	900/10,561	900/7,770 (11.58)
Total	23/56	143/1,822	123/2,314	0/1,585	0/1,304	965/6,271	0/1,403	1,231/14,699	1,231/10,407 (11.83)

*Of the 56 affected farms, 23 had samples collected for PCR analysis. All farms tested had positive results for vaccinia virus Cantagalo strain.
†No. sick animals/total no. animals (%). Cohort consisted of female cattle >24 mo of age (considered lactating cows) and all calves <12 mo of age.

related infection: vesicopustular lesions on the udder and/or teats of cows or on muzzle and/or tongue of suckling calves, fever, lymphadenopathy, and remission after ≈3 weeks. A confirmed case was any probable case that was laboratory confirmed by PCR or that had an epidemiologic link to a farm with PCR-positive results. We considered as epidemiologic links the migration of dairy workers, animal movements, and location of neighboring farms. Each inspection generated an epidemiologic investigation form by the animal health agencies of these states. Human infection was noted with a description of lesions on hands, arms, and face.

A total of 52 samples of scabs were collected from cattle on 23 farms. DNA was isolated as described (*5*) and subjected to PCR by using the EACP primers to detect the full-length hemagglutinin (HA) gene, a marker for orthopoxvirus infection (*1*,*5*,*7*). To specifically detect CTGV, we used a reverse primer that annealed to the flanking regions of an 18-nt deletion in the HA gene, a molecular signature of the CTGV-like strains from Brazil (*1*,*3*,*5*). DNA from reference CTGV (*1*) was used as positive control, and VACV-WR and cowpox virus strain Brighton Red were used as negative controls (*5*). This strategy successfully identified CTGV-like isolates, as confirmed by DNA sequencing (*5*,*7*). Thirty-five samples, representative of the 23 farms (Table 1), were positive for both orthopoxvirus (full-length HA) and CTGV infections. This result strongly suggests that CTGV was the etiologic agent of disease in these farms. For further confirmation, we sequenced the HA, K2L, and C7L genes from 6 samples, as described (*7*). All sequences showed the 18-nt deletion in the HA gene as well as the 15-nt deletion within K2L found in all CTGV-like isolates (*7*,*9*). The relatedness of the virus isolates with CTGV-like isolates was confirmed by analysis of the nucleotide identity matrix (Table 2) and phylogenetic inference (data not shown).

An epidemiologic investigation was conducted by using data available from the 56 epidemiologic investigation forms, analyzing cattle movement forms issued during this period, and interviewing farmers and animal health agency

Table 2. Nucleotide identity scores obtained for the concatenated alignment of HA, C7L, and K2L genes of Mato Grosso and Rondônia isolates and distinct vaccinia virus strains, Brazil, 2008–2010*

Strain	VACV-Cop	VACV-Lister	VACV-WR	ARAV	CTGV-MU-07	CTGV	CTGV-VSD-01	CTGV-URU-04	CTGV-URU-06	CTGV-JP-11	CTGV-JP-13	CTGV-ESP-01
VACV-Cop	ID	0.990	0.990	0.971	0.971	0.971	0.971	0.971	0.971	0.971	0.971	0.971
VACV-Lister	0.990	ID	0.991	0.972	0.972	0.972	0.972	0.972	0.972	0.972	0.972	0.972
VACV-WR	0.990	0.991	ID	0.976	0.976	0.976	0.976	0.976	0.976	0.976	0.976	0.976
ARAV	0.971	0.972	0.976	ID	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999
CTGV-MU-07	0.971	0.972	0.976	0.999	ID	0.999	0.999	0.999	0.999	0.999	0.999	0.999
CTGV	0.971	0.972	0.976	0.999	0.999	ID	0.999	0.999	0.999	0.999	0.999	0.999
CTGV-VSD-01	0.971	0.972	0.976	0.999	0.999	0.999	ID	1.000	1.000	1.000	1.000	1.000
CTGV-URU-04	0.971	0.972	0.976	0.999	0.999	0.999	1.000	ID	1.000	1.000	1.000	1.000
CTGV-URU-06	0.971	0.972	0.976	0.999	0.999	0.999	1.000	1.000	ID	1.000	1.000	1.000
CTGV-JP-11	0.971	0.972	0.976	0.999	0.999	0.999	1.000	1.000	1.000	ID	1.000	1.000
CTGV-JP-13	0.971	0.972	0.976	0.999	0.999	0.999	1.000	1.000	1.000	1.000	ID	1.000
CTGV-ESP-01	0.971	0.972	0.976	0.999	0.999	0.999	1.000	1.000	1.000	1.000	1.000	ID

*GenBank accession nos. for vaccinia virus genomes are as follows: VACV-Cop (Copenhagen strain), M35027; VACV-Lister, AY678276; VACV-WR (Western Reserve strain), NC_006998. Individual GenBank accession nos. for HA, C7L and K2L genes of CTGV and CTGV-related viruses are as follows, respectively: ARAV (Araçatuba virus) (*3*): AY523994, EF051277, EF175987); CTGV isolate MU- 07 (*7*): FJ545689, FJ545688, FJ545687; CTGV (reference sample) (*1*): AF229247, EF488959, EU528619; CTGV isolates investigated in this work VSD-01(municipality of Vale de São Domingos, MT, 2008): HQ336388, HQ336394, HQ336400; URU-04 (municipality of Urupá, RO, 2009): HQ336387, HQ336393, HQ336399; URU-06 (municipality of Urupá, RO, 2009): HQ336389, HQ336395, HQ336401; JP-11 (municipality of Ji-Paraná, RO, 2010): HQ336385, HQ336391, HQ336397; JP-13 (municipality of Ji-Paraná, RO, 2010): HQ336384, HQ336390, HQ336396; ESP-01 (municipality of Espigão D'Oeste, RO, 2010): HQ336386, HQ336392, HQ336398. HA, hemagglutinin; VACV, vaccinia virus; CTGV, vaccinia virus Cantalago strain; MT, Mato Grosso State; RO, Rondônia State; ID, identical viruses.

veterinarians. In addition to the 23 farms with positive samples, 33 other farms had epidemiologic links to CTGV-positive farms. Therefore, our data suggest that CTGV infection was the cause of all outbreaks.

Analysis showed disease prevalence among lactating cows and suckling calves to be 11.83% (1,231/10,407) (Table 1). These rates were lower than those for farms of southeastern states, which reached 50% to 80% (*10*). Factors that may account for this difference were more efficient surveillance systems in Mato Grosso and Rondônia and the notably larger herd size on Mato Grosso and Rondônia farms. In this study, 55.36% of the affected farms had ≥100 cattle (mean=253), in contrast to 90% of southeastern farms, which had ≤100 cattle (*5*,*10*). The economic losses were substantial because milk production was the major activity at 76.79% of the farms in this study; the income of 93.02% of those was solely based on dairy activities. In addition, 67.85% of the farms reported at least 1 infected dairy worker.

BR-364 is a 4,141.5-km highway with a major veterinary inspection station, the Amazon Gate, at the Mato Grosso–Rondônia border. Affected farms were mostly located along this main road connecting Mato Grosso with the Amazon region (Figure 1, panel A). Despite that, we could not establish an epidemiologic link between the outbreaks in Mato Grosso and Rondônia. In contrast, the spread and establishment of CTGV within Rondônia were evidently related to animal movement along BR-364. Outbreaks in Rondônia (Figure 1, panel B) were first reported in August 2009 after the First Rondônia Dairy Cattle Auction in the municipality of Ji-Paraná (Figures 1, 2), which is part of Rondônia's major dairy region (*11*). According to auction records, animals were not traded from other states for this event. Therefore, the circumstances of CTGV entry into Rondônia are still uncertain, but informal animal trade should be considered as well as the pre-event translocation of temporary dairy workers from Mato Grosso, as reported by some farmers.

Cattle trade during the auction launched virus spread throughout the state, which was amplified by subsequent trading and human translocation (Figure 2). Animal movement was associated with the disease spread on 48.57% of farms, whereas migrating dairy workers and neighboring farms were associated with 22.86% and 28.57% of the cases, respectively. Conversely, in Mato Grosso, 13.04% of the farms were related to the virus spread by animal movement, whereas 60.87% were associated with migrating workers. On southeastern farms, dairy workers have also been associated with virus spread, but no quantitative data have been reported (*5*,*10*). The distinct pattern in Rondônia could be related to extraordinary intrastate cattle movement. Analysis of 459,884 cattle movement forms issued in 2009 revealed an intrastate translocation of 6,875,031 cattle, which corresponded to 29.34 cattle/km^2 in contrast to 17.44 cattle/km^2 in Mato Grosso. The occurrence of new CTGV outbreaks in 2010

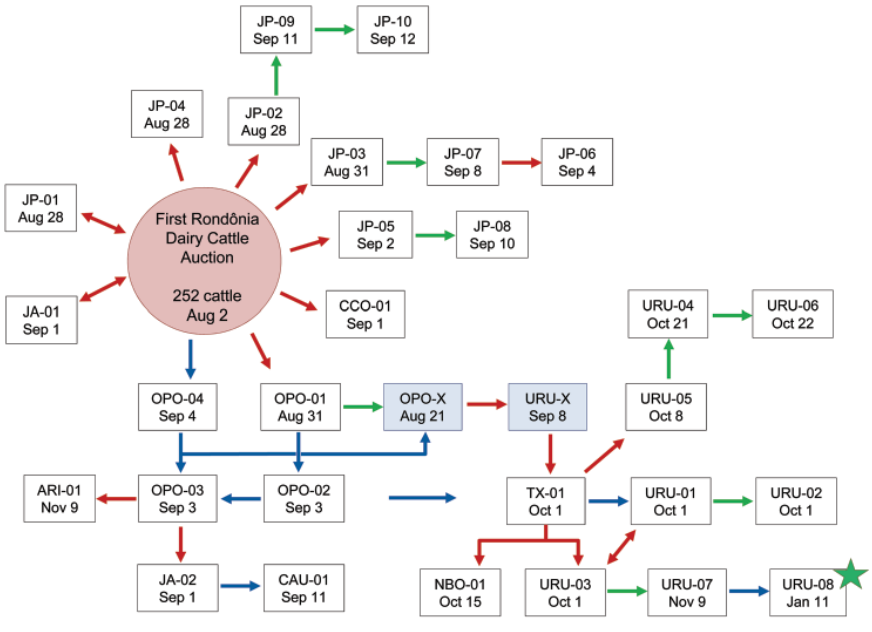

Figure 2. Spread of vaccinia virus Cantagalo (CTGV) strain infection and the epidemiologic links between affected farms in Rondônia, 2009. Two farms provided 252 cattle for the First Rondônia Dairy Cattle Auction (10°51′02.38″S, 61°59′23.93″W), which occurred in Ji-Paraná, Rondônia, on August 2, 2009. Eighty-one animals were sold to 6 farms; the remaining cattle were returned to their original owners. Dairy workers were hired as temporary workers for the pre-event period. Each farm affected by CTGV infection in 2009 is represented by a white box with date of disease notification to the Rondônia animal health agency indicated. The report usually happened 2–15 days after onset of clinical signs, but occasionally veterinarians were contacted after lesions had healed. Light blue boxes indicate farms whose owners did not report sick animals but which traded cattle with CTGV-affected farms or had close contact with them; the date of animal trading is shown. Epidemiologic links between farms are indicated by arrow colors (red for animal trade, blue for migration of workers, green for neighboring farms) but do not infer geographic distances. Farms were located in these municipalities: JP, Ji-Paraná; JA, Jaru; CCO, Cacoal; OPO, Ouro Preto D'Oeste; ARI, Ariquemes; CAU, Cacaulândia; TX, Teixeirópolis; NBO, Nova Brasilândia D'Oeste; URU, Urupá. Green star indicates a farm where animals showed clinical signs December 13, 2009, but the owner did not notify the state agency until January 11, 2010.

supports a nonsporadic pattern of infection in Rondônia, highlighting the establishment of this zoonotic infection within the state.

Conclusions

This study investigated the appearance, establishment, and spread of CTGV infection into the Amazon biome. Our findings suggest that animal movement was the main cause of virus long-distance dissemination in Rondônia, whereas the migration of dairy workers was involved in the focal spread. Animal movement plays an essential role in cross-border spread of several disorders worldwide, including bovine tuberculosis, foot-and-mouth disease, and bovine diarrhea viruses (*12–15*). We suggest animal movement is also a notable risk factor for CTGV dissemination. According to the inspection station database at the Amazon Gate, 287,906 cattle were moved into Rondônia through BR-364 in 2008–2009, of which 12,501 stayed in Rondônia. Attention is needed to address the westward spread of this zoonosis into the Amazon biome, toward the third largest dairy region of Rondônia surrounding Porto Velho (Figure 1, panel B). Therefore, new regulations and intensified surveillance should be implemented to restrain CTGV spread, particularly in dairy regions with elevated rates of cattle trade for heifer replacement.

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Vaccinia Virus Infections in Martial Arts Gym, Maryland, USA, 2008

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Vaccinia virus is an orthopoxvirus used in the live vaccine against smallpox. Vaccinia virus infections can be transmissible and can cause severe complications in those with weakened immune systems. We report on a cluster of 4 cases of vaccinia virus infection in Maryland, USA, likely acquired at a martial arts gym.

Vaccinia virus (VACV) is the virus used in the live vaccine against smallpox. Smallpox was declared eradicated by the World Health Organization in 1980 (*1*), and routine childhood smallpox vaccination ceased after 1972 in the United States. Since 2002, smallpox vaccinations have again been administered to some military personnel and health care workers, and they continue to be recommended for laboratory workers who work with nonattenuated orthopoxviruses (*2*). VACV infections are transmissible and can cause severe complications in those with weakened immune systems (*3*). We report a cluster of community-acquired VACV infections at a martial arts gym in Maryland, USA.

Case-Patients

In July 2008, the Michigan Department of Community Health (MDCH) Bureau of Laboratories reported a suspected orthopoxvirus infection to the Centers for Disease Control and Prevention (CDC). In the affected person, a 26-year-old male resident of Maryland , multiple pustules had developed on the arm, chin, and back of the knee on June 16 (Table; case-patient 1). He sought treatment after a fever and headache developed on June 19 and was advised to go to an emergency room if his fever worsened (Figure 1). His condition did not improve, and he was hospitalized in Maryland on June 23. His lesions were umbilicated pustules ≈0.5 cm in diameter with similar morphologic features (Figure 1). The cause of his illness remained undetermined, and he was discharged on June 26 after defervescence. Contact precautions were employed during the hospitalization; however, the patient was not isolated in a negative-pressure isolation room.

Lesion samples were collected on June 24 and forwarded by the Maryland hospital to a virology reference laboratory in Michigan for testing. The samples were negative by PCR for varicella, adenovirus, and herpes simplex virus. Cytopathic effect suggestive of an orthopoxvirus was noted in MRC-5, A549, and primary rhesus monkey kidney cells, and the samples were forwarded to the MDCH laboratory for further testing. On July 4, the MDCH laboratory confirmed the presence of orthopoxvirus DNA in the lesion sample using an orthopoxvirus (nonvariola) and an orthopoxvirus generic real-time PCR (*4*). The specimens were sent to CDC for confirmation and virus species identification. Real-time PCRs designed to differentiate orthopoxvirus species were performed, and samples were positive for VACV DNA but not for monkeypox virus DNA (*5*) (Table).

The Maryland Department of Health and Mental Hygiene was contacted on July 3 and, in collaboration with the Montgomery County Department of Health and Human Services, began an investigation. The patient was asked whether he recently received smallpox vaccination or had history suggestive of exposure to orthopoxviruses such as monkeypox virus (i.e., contact with animals, recent international travel). He reported having neither; however, his wife and child had returned from a trip to Brazil 2 weeks before his illness. Human VACV infections caused by contact with infected dairy cattle occur in regions of Brazil (*6*). His wife only visited an urban area in Brazil, reported no contact with farm animals, and reported no fever or rash.

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[1]These authors contributed equally to this article.

Table. Characteristics of 4 cases of vaccinia infection at a martial arts gym, Maryland, USA, 2008*

Case-patient no.†	Age, y/sex	Date of onset	Rash features	Initial diagnosis	PCR	Serologic results
1	26/M	Jun 16	Pustules on face, arm, back of knee	Unknown viral exanthem	+	NA
2	28/M	Mid–late Jun	Vesicles on right forearm	None	Weak +	IgM+ (0.243), IgG+ (0.116)
3	31/M	Jun 25	Unknown presentation	MRSA‡	NA	IgM+ (0.389), IgG+ (0.227)
4	31/M	Late Jun/early Jul	Unknown presentation	MRSA‡	NA	IgM+ (0.137), IgG+ (0.2195)

*Ig, immunoglobulin; NA, not applicable; MRSA, methicillin-resistant *Staphylococcus aureus*.
†For purposes of this investigation, a case-patient is defined as a person whose clinical samples are positive for vaccinia virus DNA by PCR, or a person with lesions or a rash (macular, papular, vesicular, or pustular) and whose serum sample was positive for anti-orthopoxvirus IgM antibodies (indicative of a recent orthopoxvirus exposure). No case-patients had received a previous smallpox vaccination.
‡Clinically diagnosed, no laboratory confirmation.

Sequence analysis of a 160-bp fragment of the hemagglutinin gene from the virus isolate was performed at CDC to determine whether the VACV strain originated from smallpox vaccine or from a strain that occurs naturally in Brazil. The isolate matched the strain used in the ACAM2000 smallpox vaccine and was distinctive from known Brazilian VACV (Figure 2). ACAM2000 is the second-generation smallpox vaccine, which replaced Dryvax in January/February 2008 (*7*).

The patient reported belonging to a martial arts gym; he reported having several military personnel as recent sparring partners before the onset of his illness. He also reported that a recent sparring partner had exhibited a rash around the same time. This person, a 28-year-old man (case-patient 2), was contacted and described having a 4-day rash on his right forearm in mid to late June with no systemic symptoms (Figure 1). Samples of his serum, collected 2–3 weeks after lesion onset, were sent to CDC for testing and showed modestly elevated levels of anti-orthopoxvirus immunoglobulin (Ig) M antibodies (Table). Scab material tested at CDC was weakly positive for orthopoxvirus DNA by using the orthopoxvirus nonvariola and orthopoxvirus (generic) PCRs.

In the absence of an explanation for these 2 VACV (ACAM2000) infections, Maryland public health officials launched an investigation at the gym to identify additional cases and pinpoint the source of infection. Approximately 400 surveys were distributed to gym members through email and by hand at the gym. Members were asked whether they had any recent skin lesions similar to those

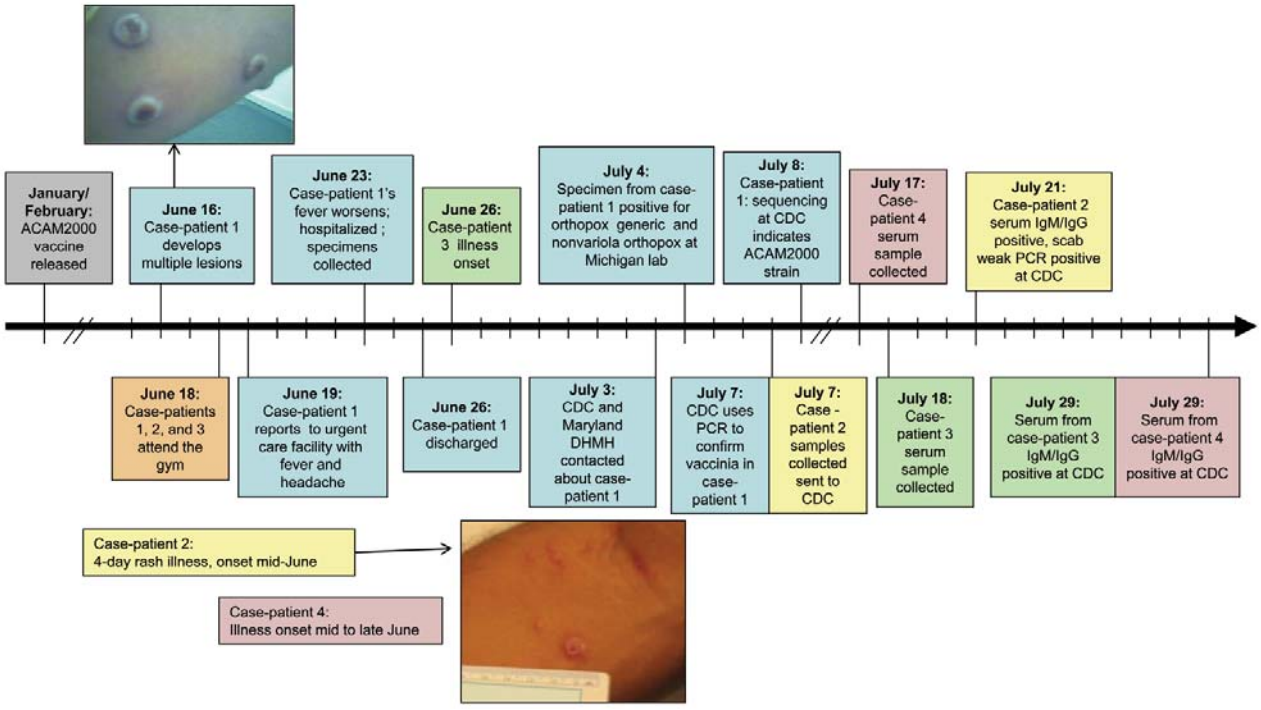

Figure 1. Timeline of the vaccinia cluster, Maryland, USA, 2008. The photo of case-patient 1's skin lesions was taken on ≈day 8 of illness (courtesy of R. Reddy). The photo of case-patient 2's skin lesions was taken ≈3 weeks after lesion onset (courtesy of K. Russo). Blue shading, case-patient 1; yellow shading, case-patieint 2; green shading, case-patient 3. CDC, Centers for Disease Control and Prevention; Ig, immunoglobulin; DHMH, Department of Health and Mental Hygiene.

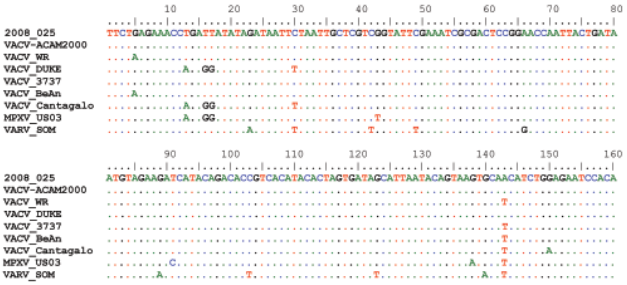

Figure 2. Partial DNA sequence alignment of the hemagglutinin gene. Case-patient 1's isolate sequence is displayed at the top (2008–025). Dots in the alignment indicate identical nucleotides at that position. The reference sequences shown: current smallpox vaccine strain (VACV_ACAM2000), a commonly used laboratory vaccinia strain (VACV_WR), Dryvax vaccinia strains (VACV_Duke and VACV_3737), natural Brazilian vaccinia isolates (VACV_BeAn and VACV_Cantagalo), a 2003 US monkeypox outbreak isolate (MPXV_US03), and a variola virus isolate (VARV_SOM). Reference GenBank accession nos., AY313847, NC_006998, DQ439815, DQ377945, DQ206442, AF229247, DQ011157, and DQ437590, respectively.

shown in an attached photo. They were asked whether they had recently received a smallpox vaccination or had contact with someone recently vaccinated.

Ninety-five gym members responded to the survey. Several reported having received a smallpox vaccination previously, but none reported vaccination within the prior 2 months. Thirteen gym members reported skin lesions or rash but no recent smallpox vaccination. Two of these persons (case-patients 3 and 4) were clinically diagnosed with methicillin-resistant *Staphylococcus aureus* (MRSA) by health care providers in late June and early July. Both attended the gym on the same day as case-patient 1 (Figure 1). Serum samples were collected from these men 3–4 weeks after lesion onset and sent to CDC for testing. Both had elevated levels of anti-orthopoxvirus IgG and IgM antibodies, indicative of a recent exposure (Table).

Maryland public health officials reviewed cleaning protocols at the gym. They determined that equipment and pads were cleaned at least twice daily (stemming from a concern about MRSA transmission) and that appropriate cleaning products were being used.

CDC identified 5 civilian clinics that had received ACAM2000 vaccine since late February in the Maryland area. These clinics reported having vaccinated 65 persons; none were members of the martial arts gym. The Military Vaccine Agency (Milvax) cross-checked its list of recent military vaccinees against the gym member list since late February. Although several of those identified as being vaccinated had an association with the gym, they were either not currently gym members or were not at the gym during this period. The source of virus introduction into the martial arts gym remains unknown. No further infections have been identified among gym members or health care workers exposed to case-patients.

Conclusions

This cluster of community-acquired VACV infection was possibly the result of sequential person-to-person spread of virus through direct physical contact, although transmission through fomites cannot be ruled out. The ultimate source-person responsible for introducing the virus into the gym was not identified, but given the limited time that ACAM2000 had been available to providers in the region (late February 2008), the most likely source was a recent vaccinee. None of the current gym members were known to have been vaccinated within the 4 weeks before illness onset of the first case-patient. Unrecognized transmission of VACV among gym members may have been ongoing over several months.

Multiple cases of VACV infection caused by secondary transmission have been noted recently (*8–13*). Materials such as towels and bedding used by the vaccinee should be treated as potential fomites and should not be shared with others (*14*). To our knowledge, this is the first reported cluster of community acquired VACV in which an obvious source-person was not identified. This cluster highlights the need to reinforce transmission precautions to recent vaccinees and indicates that physicians should include VACV infections on the differential of vesiculopustular rash lesions and take appropriate infection control precautions, even in the absence of a known exposure to smallpox vaccine.

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Ms Hughes is a health scientist with the Poxvirus and Rabies Branch, Centers for Disease Control and Prevention. Her primary research interest is the epidemiology of various poxvirus infections.

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Hepatitis A Virus Vaccine Escape Variants and Potential New Serotype Emergence

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Six hepatitis A virus antigenic variants that likely escaped the protective effect of available vaccines were isolated, mostly from men who have sex with men. The need to complete the proper vaccination schedules is critical, particularly in the immunocompromised population, to prevent the emergence of vaccine-escaping variants.

In areas where hepatitis A has low to moderate endemicity, introduction of the virus occurs through consumption of imported foods, traveling, or through immigration flows (*1*–*3*). Men who have sex with men (MSM) comprise a high-risk group for hepatitis A, and several outbreaks affecting this group have been reported across Europe (*4*). To prevent the spread of infection, since 1999, vaccination programs have been implemented among preadolescents in the Catalonia Autonomous Community of Spain.

Despite some degree of nucleotide heterogeneity at the capsid region of hepatitis A virus (HAV) (*5*,*6*), there is not an equivalent degree of amino acid variation (*7*). HAV replicates as complex dynamic mutant distributions or quasispecies (*8*) and thus the high degree of conservation of the capsid amino acid sequences among independent strains must be the result of negative selection on newly arising mutants. So far, a single serotype of human HAV has been recognized, which suggests that severe structural constraints occur in the capsid that prevent the more extensive substitutions necessary for the emergence of a new serotype. Indeed, negative selection of replacements affecting residues encoded by rare codons of the capsid surface has been documented, indicating a critical role played by such rare codons (*9*). Since these residues are located quite near or even at the epitope regions, the need to maintain such rare codons might prevent the emergence of new serotypes (*9*). We have recently noted that fine-tuning translation kinetics selection, or the right combination of preferred and rare codons in the capsid coding region, is necessary to get regulated ribosome traffic to guarantee the proper capsid folding (*10*). In this context, it seems quite unlikely that a new serotype will emerge, although the emergence of new variants is not impossible if the virus population is forced through bottleneck conditions such as immune selective pressures. We investigated the presence of antigenic variants among sporadic and outbreak cases of hepatitis A.

The Study

We molecularly characterized 128 HAV strains isolated during 2005–2009 in Catalonia from patients with both sporadic (n = 37) and outbreak (n = 91) cases (online Technical Appendix Figure 1, www.cdc.gov/EID/content/17/4/734-Techapp.pdf) based on their viral protein 1 (VP1) region (*7*). Deduced amino acid sequences were compared with those of HM-175 and GBM strains (GenBank accession nos. M14707 and X75215, respectively) and constituents of 2 of the commercial HAV vaccines, HAVRIX (GlaxoSmithKline, Rixensart, Belgium) and Avaxim (Sanofi-Pasteur, Paris, France), respectively. Six amino acid replacements, which have not been previously described, were detected (Table 1). Two were semiconservative replacements, V1171A and A1280V, and the other 4 were nonconservative, V1166G, Y1181S, R1189T, and A1280E. The replaced amino acids were located in a refined 3-dimensional computer model of the HAV protomer (*11*), and their relative distances to residues 1102, 1171 and 1176, constituents of the immunodominant site (*12*), and to residue 1221, constituent of the glycophorin A binding site epitope (*13*), were used as markers of the potential antibody-escaping phenotype. All replaced positions were located at (1171) or around (1166, 1181, 1189, 1280) the viral immunodominant site near the 5-fold axis (Figure 1), and thus strains bearing these replacements might be considered antigenic variants. In a previous study, several escape mutants to K34C8 monoclonal antibody (MAb), which recognizes the immunodominant site, were isolated (*9*). Among these mutants, 2 were defined by replacements W1170C (C6) and A1187P (P29), which were located very close to the mutated residues detected in this study (Figure 1). Residue 1170 is located contiguous to residue 1171 and close to residues 1280 and 1181. Additionally, residue 1187 is in close contact with residue 1189. Since HAV natural isolates cannot be grown in vitro, C6 and P29 monoclonal

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Table 1. Amino acid substitutions in the VP1 protein observed in different strains isolated during the present study*

Strain	Replacement	Position*
MSM08-09-219	V → G	1166
MSM08-09-186	V → G	1166
	V → A	1171
BCN60	Y → S	1181
MSM08-09-ClusterE	R → T	1189
MSM08-09-144	A → V	1280
BCN31	A → E	1280

*Position strain HM175 (GenBank accession no. M14707). The first digit refers to the viral protein, i.e., 1 for VP1, and the following 3 digits refer to the amino acid position in the protein.

antibody–resistant (MAR) mutants were used to mimic the behavior of the naturally isolated variants in neutralization assays with antivaccine serum specimens. Results proved that mutant C6 is resistant to both antivaccine serum, as well to convalescent-phase serum, whereas mutant P29 is partially resistant to serum generated with Avaxim vaccine (Table 2).

Of the 6 antigenic variants isolated (Table 1), 4 were obtained during an outbreak among MSM in 2008–2009. Although the number of reported cases of this outbreak was of 186, the number of molecularly analyzed samples, i.e., 66, was similar to that of the rest of analyzed samples, i.e., 62 (including other small outbreaks as well as sporadic cases). Phylogenetic analysis of antigenic variants in the MSM group suggested that they originated from a single patient (online Technical Appendix Figures 1, 2), and thus 4 variants (6% of all isolated strains) represent quite an important number for such an antigenically stable virus. In contrast, the multiple origins of the strains of the general group (42 phylogenetically distinguishable strains; online Technical Appendix Figure 1) did not correlate with higher numbers of antigenic variants: only 2 were detected (3% of all isolated strains). An intriguing issue is why so many variants of the immunodominant 5-fold site arose during the MSM 2008-2009 outbreak, considering the low fitness shown by MAR mutants with replacements around this site. In particular, C6 and P29 mutants were rapidly outcompeted by a wild-type strain in the absence of antibodies, and although they were able to overcome the wild-type virus in the presence of the K34C8 MAb, it was only after a slow process (Figure 2), indeed indicating a very low fitness. This kind of mutant can only be selected throughout bottleneck events, such as the ingestion of minute amounts of viruses able to float some variants, unlikely in high-risk practices of HIV-positive patients whose viral load in stool may be as high as 10^{11} genome copies/g, or the ingestion of a considerable amount of viruses by patients with low IgG levels, who are unable to completely neutralize the infecting virus, thus allowing the viral population to replicate in the presence of antibodies. In fact, 4% (8/186 case-patients) of the MSM 2008–2009 outbreak patients had been vaccinated. However, in only 1 case was the vaccine administered during childhood following the complete dose schedule. In 5 cases, the patients had received only 1 dose of the vaccine during the 6-month period before any symptoms of infection, and the remaining 2 had received only 1 dose of the vaccine long before the infection. Five of these 8 patients were HIV positive. An incomplete vaccination schedule in an immunocompromised host could lead to a situation of only partial protection, providing suitable conditions for the emergence of an antigenic variant. Unfortunately, samples

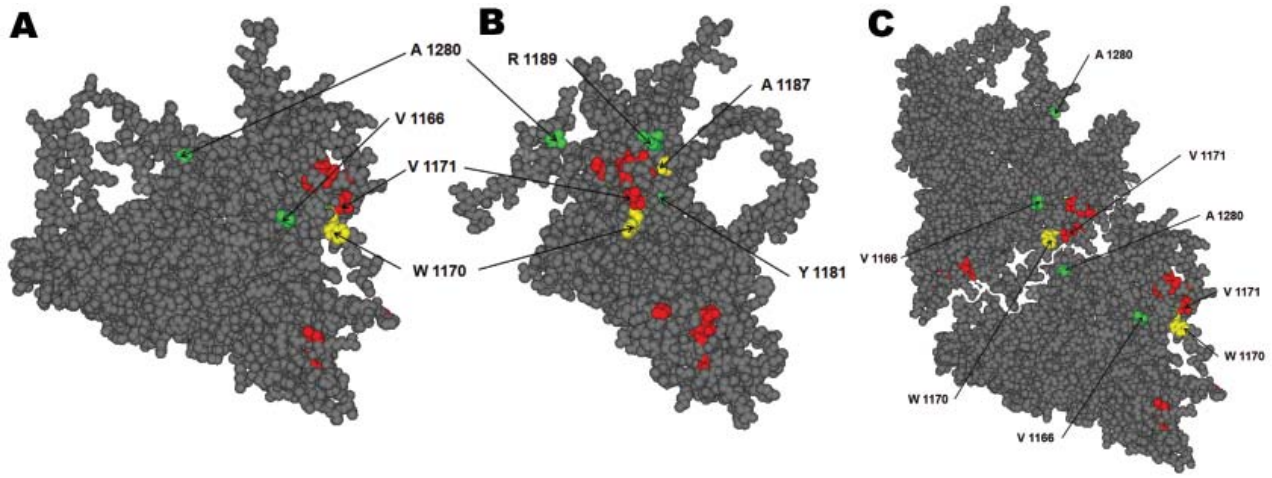

Figure 1. Hepatis A virus protomer model (*11*; refined by Ming Luo, University of Alabama, Birmingham, AL, USA), which includes the locations of all of the substituted residues in viral protein 1 detected in the isolated variants during 2005–2009. A) Front view of the external surface. B) Lateral view. C) View of 2 adjacent protomers, showing the close contact of residues 1171 and 1280. Red, residues forming the immunodominant site; yellow, residues substituted in monoclonal antibody–resistant mutants C6 (W1170C) and P29 (A1187P); green, residues substituted in the identified natural variants. The amino acid substitution V1171A detected in 1 variant is shown in red because this residue belongs to the immunodominant site.

Table 2. Neutralization assays of K34C8 MAb-escape variants that showed replacements at the same or very close positions as the mutated positions in the naturally-selected field variants isolated during 2005–2009*†

Mutant (position replaced)	log N_t/N_0 vaccine serum (HAVRIX)	log N_t/N_0 vaccine serum (Avaxim)	log N_t/N_0 convalescent-phase serum (HCS2)	log N_t/N_0 MAb K34C8
C6 (1170)	–0.08 ± 0.14	–0.08 ± 0.14	–0.02 ± 0.04	–0.08 ± 0.14
P29 (1187)	–0.70 ± 0.09	–0.30 ± 0.19	–0.70 ± 0.07	–0.37 ± 0.19
D23 (1217)	–0.88 ± 0.02	–0.54 ± 0.01	–0.61 ± 0.07	–0.58 ± 0.12
HM175/43c	–0.69 ± 0.09	–0.60 ± 0.05	–0.65 ± 0.05	–0.61 ± 0.10

*Assays were performed by using vaccine and convalescent-phase serum samples as well as K34C8 MAb. MAb, monoclonal antibody; HAVRIX, HAVRIX vaccine (GlaxoSmithKline, Rixenart, Belgium); Avaxim, Avaxim vaccine (Sanofi-Pasteur, Paris, France).
†Following the model of the hepatitis A virus protomer of Ming Luo (Figure 1). Three neutralization assays were performed with each antivaccine serum sample, the convalescent-phase serum sample, and the MAb K34C8. As controls, neutralization of the D23 H7C27 MAb escape variant (*9*) as well as that of the HM175/43c wild-type strain, was also measured. The highest dilution showing a log N_t/N_0 = –0.60 (75% neutralization) of the wild-type strain was used to test the variants; N_t, the viral titer after neutralization; N_0, the initial titer. Neutralization limits were the following: log Nt/N_0>–0.26 (<45%) for resistant variants, –0.26>log Nt/N_0>–0.60 (45%–75%) for partially resistant variants, and log Nt/N_0<–0.60 (>75%) for sensitive variants (*9*).

from these vaccinated patients were not available; however, it is obvious that the vaccinees likely contributed to the selection of such antigenic variants at a population scale. Because in situations of no competition with the wild-type virus, the MAR mutants replicate perfectly well, it may be inferred that the natural variants, once they are selected in an improperly vaccinated HIV-positive person, may spread to other, properly vaccinated persons.

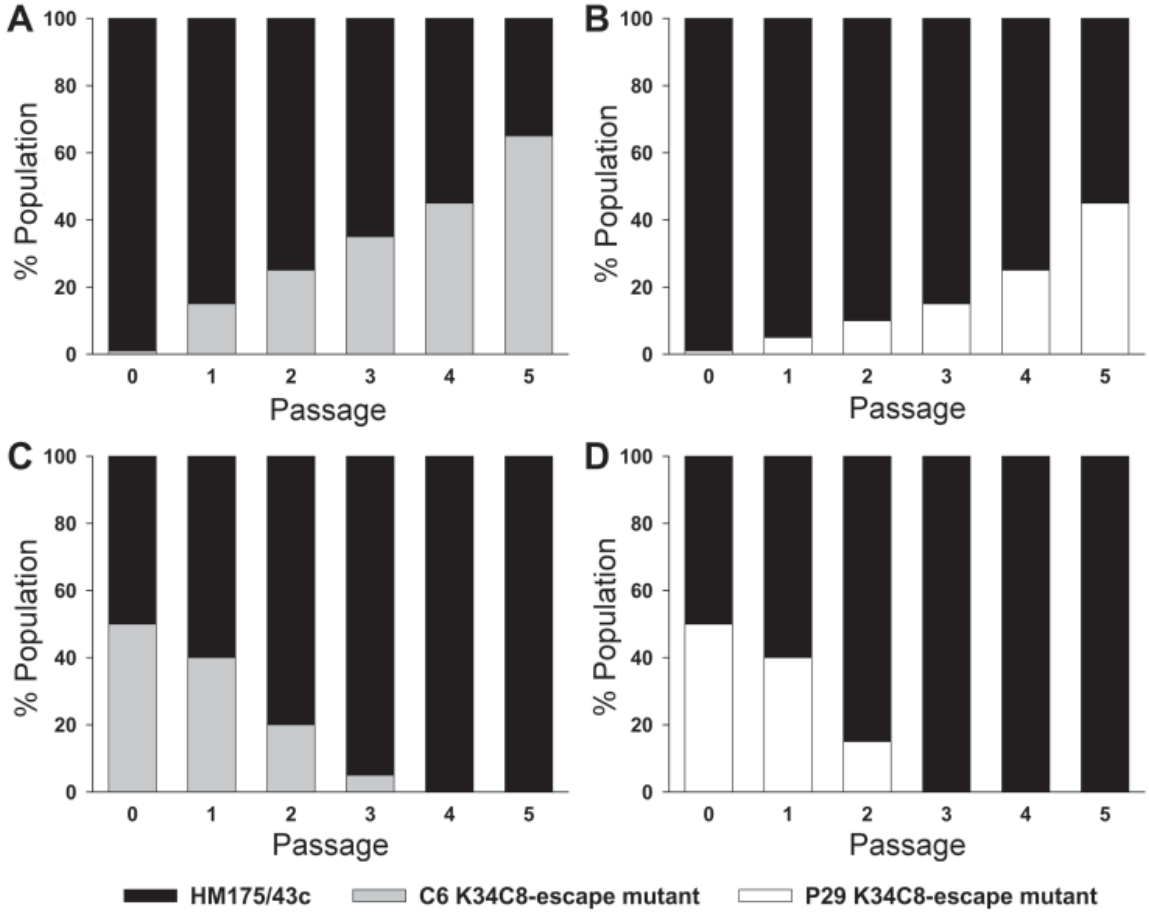

Figure 2. Growth competition experiments. Monoclonal antibody–resistant (MAR) mutants C6 (W1170C) and P29 (A1187P) were grown in competition with the HM175/43c (wild-type virus) in the presence (A,B) or in the absence (C,D) of the monoclonal antibody (MAb) K34C8. The MAR/wild-type ratios were 1:100 (10^4 50% tissue culture infective dose [$TCID_{50}$] units of MAR mutants mixed with 10^6 $TCID_{50}$ units of the wild-type virus in the presence of the K34C8 MAb) and 1:1 (10^6 $TCID_{50}$ units of MAR mutants mixed with 10^6 $TCID_{50}$ units of the wild-type virus in the absence of antibodies). In the competition experiments performed in the presence of antibodies, the initial viral mixtures as well as the viral progenies were neutralized with the MAb prior each infection passage. The proportion of mutant and wild-type phenotypes at each passage was inferred from the chromatogram of the consensus sequences and using as marker mutations W1170C and A1187P in C6 and P29 MARs, respectively (*9*).

Conclusions

Isolation of so many variants in a single outbreak among the MSM population, in a virus presenting such severe genomic and structural constraints, emphasizes the need to target this community with more effective information on risky sexual practices and vaccination programs. Additionally, and particularly among HIV-positive MSM, efforts should be made to completely accomplish the vaccination schedule, due to their lower level of immune response (*14*,*15*). An additional concern is that this impaired response may contribute not only to a lower protection of the vaccinee but also to the emergence of antigenic variants. In the analyzed MSM 2008–2009 outbreak, 4 variants were isolated that were located at or very close to the immunodominant site as well as to residues substituted in 2 MAR mutants showing a phenotype of resistance to the protection offered by commercial vaccines. Thus, a similar behavior of the natural variants can be postulated, and if this is the case, a new serotype could emerge.

Acknowledgments

We are indebted to Josep Costa and Bertram Flehmig for providing serum specimens from patients who had seroconverted to HAVRIX and Avaxim vaccines, respectively.

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Edward Jenner Museum

William Foege

Ground zero for vaccinology is Edward Jenner's home in Berkeley, England. Here Edward Jenner worked, studied, and practiced as a country doctor—and later dominated a decade of my life! Jenner knew that poets talked about the nice complexions of milkmaids, and he heard a milkmaid say she was immune to smallpox because she had acquired cowpox. He came to believe in the protective effects of cowpox after careful review of the experiences of milkmaids during smallpox outbreaks. He spent a dozen years observing before he experimented with the transfer of cowpox from a lesion on the hand of Sarah Nelms, a milkmaid, to 8-year-old James Phipps, the son of a local laborer, in May 1796. Although Jenner had no concept of viruses, immune systems, or vaccinology, he used science to help imitate what he saw happening in nature.

The Jenner home (Figure 1) in Berkeley has served as a museum (www.jennermuseum.com) for the past 25 years, and with both facts and artifacts continues to tell the story of Jenner's life and discoveries. In September 2010, Sarah Parker, the museum's director, provided us with a day of her time to show what has been done, to discuss what is planned, and to answer questions about the museum and Dr. Jenner. The only part of the house off limits to our group was Jenner's study, which had to be viewed through a glass partition but was fully visible. While he was sitting at this desk in 1823, writing up notes from a house call to verify the death of the coroner, Jenner had a stroke. He was then carried upstairs to his bedroom and never regained consciousness. On one wall is a bookcase that is standing open as it was at the time of his death.

The home provides a fascinating view into the life of a village doctor in the early 19th century. I was struck by a work table set up as it would have been at that time. The light through the window produced shadows that made an intriguing picture (Figure 2), which would have been spoiled by the use of a flash.

The building is a maze of stairways and doors. Although Jenner's medical office was actually down the street from the house, in addition to that office, he had work areas at home where he studied a variety of natural phenomena. One irony of Jenner's life is that he was admitted to the Royal Society not on the basis of his smallpox discoveries, but because of his observations that cuckoos laid their eggs in other birds' nests.

At one point Sarah Parker asked if we had any idea regarding the identity of a fan-shaped object standing upright in front of a fireplace. I said I didn't know, of course, but it looked like the scapula of a whale. She looked dejected, because that was actually the correct answer. Evidently, at some point, a whale had beached near Berkeley, and Jenner, with his usual curiosity, dissected it, keeping the scapula for his personal collection.

We even explored the attic, which contains materials from 200 years ago that have not yet been catalogued or studied. We viewed, through windows, the water collection system used for the house. The first two floors of the house

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Figure 1. The Edward Jenner home, Edward Jenner Museum, Berkeley, Gloucestershire, England.

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Figure 2. Work table, Edward Jenner Museum, Berkeley, Gloucestershire, England.

were used by Jenner and his family while servants lived in the attic. This provided another fascinating insight to a social dynamic that was common then but is difficult to imagine now.

His garden was a joy to observe. At one end of the garden is the thatched roofed house (Figure 3) where poor children would assemble on 1 day each week, and Jenner would vaccinate them at no cost. On the other side are the grape vines that he planted. The vines are unusual looking because the roots grow outside but the stems are then passed into a greenhouse, which assists in the maturation and the collection of the grapes. We were allowed to eat grapes from these vines.

Next door to the house is a church with a burial ground containing the graves of many of the people who figure into Jenner's story. Twenty-six years ago, before the museum opened, my son Michael and I stopped in this churchyard on our return from a trip to India. We were fortunate to encounter a vicar who was retired but interested in Jenner. He had returned to conduct a funeral, and we met him by chance. He spent 3 hours recounting the history of Jenner and the community of Berkeley.

While standing in the church talking with us, the vicar noticed a woman quietly waiting for him to finish. He asked if he could help her, and she said she was looking for the grave of an ancestor, Sarah Nelms. I asked, "You mean the milkmaid?" She was as surprised by my question as I was at hearing the name.

Also close by is Berkeley Castle. It has been inhabited by the Berkeley family since it was first built in the twelfth century and is said to be the oldest castle in the United Kingdom inhabited continuously by the same family. Jenner's need to tend the Berkeley family is believed to have interrupted his smallpox work, and this obligation could account for the 2-year delay in publishing his paper after the vaccination of James Phipps.

By great fortune, we had the chance to meet Professor Gareth Williams, internist, expert on diabetes and obesity, and a medical college dean and author. He lives within biking distance of the museum and had decided to use a sabbatical to write a book on Jenner and smallpox (Angel of death: the story of smallpox. Basingstoke [UK]: Palgrave-MacMillan; 2010). His interest stemmed from noticing the

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Figure 3. Cottage used by Edward Jenner for vaccinating children, Edward Jenner Museum, Berkeley, Gloucestershire, England.

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Figure 4. Painting of a cow, Edward Jenner Museum, Berkeley, Gloucestershire, England.

Jenner name in the history of his church. Jenner's father and 2 brothers were clergymen. Professor Williams also has a reputation for baking cakes for various occasions, and he appeared at the museum with a cake for our meeting!

The museum continues to expand but runs on a shoestring budget. (I say this for the benefit of anyone who has ideas on funding.) Ms. Parker has developed many displays in the past few years and has plans for the future, all depending on resources. She is even considering restoring part of the building so it can serve as a bed-and-breakfast inn for people who might want to spend a night in Jenner's house. In addition, the recently formed Edward Jenner Society (www.edwardjennersociety.org), which is dedicated to vaccinology, uses the Jenner home for its meetings.

There is a legend regarding ghosts in the building, and a photograph is displayed in the house that supposedly shows a ghost in the background. Scientists, of course, do not give much credence to such stories. I did try to take a picture of a painting of the cow that had infected Sarah Nelms with the cowpox virus, which in turn was transferred to James Phipps. The first picture had too much glare, so I took a second one less than a minute later. I was totally surprised to see the image of a woman directly below the cow's stomach (Figure 4). Because we know of no pictures of Sarah Nelms, the milkmaid involved, it would be tempting to believe it was she. But we have to make some assumptions on the identity of the person in the second photo!

Much more could be told about the insights to be gained from this museum, but instead I recommend a visit. Although it is a natural place to visit if one is interested in vaccines, I firmly believe that it is also ground zero for public health. The modern public health movement can be dated to May 14, 1796, when a vaccine for smallpox became available.

Dr Foege is a former director of the Centers for Disease Control and was active in the global campaign to eradicate smallpox. He currently is a senior fellow in the Global Health Program of the Bill & Melinda Gates Foundation, where his work focuses on broadening public awareness of the issues of child survival and development, population, preventive medicine, and public health leadership.

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Acute Cytomegalovirus Pneumonitis in Patient with Lymphomatoid Granulomatosis

To the Editor: Lymphomatoid granulomatosis (LYG) involves a B-cell lymphoproliferative process associated with Epstein-Barr virus (*1*). The disease is characterized predominantly by lung involvement, and the pathologic findings show an angiocentric pattern with lymphoid cell clustering. Patient median survival time is ≈14 months (*2*). Treatment commonly consists of corticosteroids and cyclophosphamide, but a literature review did not demonstrate any benefit from corticosteroid therapy, cytotoxic chemotherapy, or radiation (*3*). Rituximab, a monoclonal antibody, targets a B-cell surface molecule, CD20. Recently, several case reports have been published about the effectiveness of rituximab for LYG (*4*,*5*).

We report acute cytomegalovirus pneumonitis in a patient with LYG. In May 2005, a 40-year-old woman with no history of systemic disease had experienced intermittent dry cough for 1 month. Her cough was not associated with any specific environmental exposure or location. Computed tomographic (CT) scan of the chest showed bilateral multiple lung nodules, and a lung biopsy sample showed lymphohistiocytic and lymphoplasmacytic cell infiltrates with some fibrous material. Prednisolone was prescribed for suspected cryptogenic organizing pneumonitis, but her symptoms improved only partially.

One year later, her symptoms worsened. Chest CT scan showed more nodules in both lungs (Figure, panel A). A repeat lung biopsy sample showed large abnormal lymphoid cells clustering in pink-white nodules around and within blood vessel walls. The abnormal lymphocytes were positive for CD20 and Epstein-Barr virus–encoded RNA. The patient was HIV negative, and her bone marrow showed no evidence of lymphoma. Abdominal and pelvic CT scans did not show other abnormal lymph nodes or organ involvement. LYG was diagnosed.

The patient's condition did not respond well to induction chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisone, and her dyspnea persisted. One week later, rituximab was prescribed; dyspnea dramatically improved after 1 day, and she could tolerate room air without oxygen support. However, 1 day later, severe respiratory distress with hypoxemia suddenly occurred. Although chest CT scan disclosed dramatic resolution of the previously found nodular lung lesions, it also showed newly developed interstitial ground glass opacities (Figure, panel B). A diagnosis of interstitial pneumonitis was considered. Serum cytomegalovirus (CMV) DNA quantification by pp65 gene PCR was performed; the amount was high, up to 2×10^5 copies/mL. Acute CMV pneumonitis was diagnosed. Although the patient received mechanical ventilatory support, CMV intravenous immunoglobulin administration, and ganciclovir therapy, she died 5 days after the onset of acute CMV pneumonitis.

CMV pneumonitis is a common presentation of CMV disease in immunocompromised patients. Host factors, such as presence of cancers or compromised immune function, play a major role in determining pathogenicity of the virus. Respiratory failure is the leading cause of death among patients with rapidly progressive interstitial pneumonitis related to CMV, especially among recipients of renal and bone marrow transplants (*6*). The patient reported here received chemotherapy and steroids; both treatments would render the patient immunocompromised, a condition that may lead to such a rapidly progressive course.

This patient had no identifiable concurrent illnesses that might have been associated with compromised immunity or development of LYG. However, fatal CMV pneumonitis, a complication usually associated with extremely impaired immunity, developed. The case suggests that, even without an identifiable immunocompromised condition, a patient with LYG should be considered an immunocompromised host, and

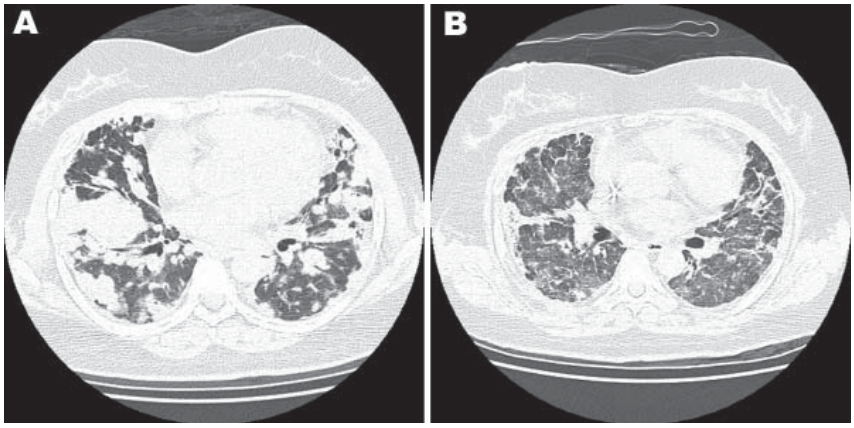

Figure. Chest computed tomography scan of a 40-year-old woman with no history of systemic disease. A) Bilateral multiple lung nodules and lymphomatoid granulomatosis were diagnosed after lung biopsy. B) After rituximab treatment, the prior nodular lung lesions decreased dramatically, but newly developed interstitial ground glass opacities appeared.

rituximab or other immunosuppressive treatments should be prescribed cautiously; the possibility for rare complications should be recognized.

Because of massive ablation of humoral immunity, the relationship between rituximab and virus infection has been addressed, including varicella–zoster infection, parvovirus B19 infection, and CMV reactivation (*7*). In immunocompromised patients, rituximab might lead to higher risk for virus infection. This issue has been addressed with HIV/AIDS patients with high-grade B-cell lymphoma for whom rituximab is not generally recommended because B-cell ablation could result in more opportunistic infections. For LYG, increased frequency is associated with both congenital and acquired immunodeficiency, such as X-linked lymphoproliferative syndrome, Wiskott–Aldrich syndrome, and HIV/AIDS in which T-cell surveillance is deficient (*8*). Thus, for a patient with LYG whose immune system might be abnormal (*9*), the risks associated with rituximab therapy should be considered the same as the risks for HIV/AIDS patients, and the risk for viral infection or reaction to rituximab should be recognized, particularly in areas where CMV seropositivity in the population is high (*10*). In addition, especially for adult and elderly patients, the gradual increase of CMV seroprevalence with age should be recognized (*10*). Moreover, the patient reported here had previously received cytotoxic drugs as well as maintenance steroid therapy, both of which contributed to a severely compromised immune system. These factors may have led to her acute CMV pneumonitis after receipt of rituximab.

In conclusion, the potential for acute CMV reactivation should recognized during use of rituximab to treat patients with LYG. During rituximab treatment of LYG, routine monitoring for CMV reactivation and other viral infections is warranted.

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Livestock-associated *Staphylococcus aureus* in Childcare Worker

To the Editor: Carriage of *Staphylococcus aureus* sequence type (ST) 398 has primarily been reported as occurring among persons in contact with livestock, including swine and cattle (*1*,*2*). This association has given rise to the characterization of this strain as livestock associated (*3*). However, ST398 colonization or infection in persons lacking identified livestock-associated risk factors have been reported (*4*,*5*). We report ST398 colonization in a childcare worker in Iowa, USA.

As part of a surveillance study of *S. aureus* carriage in child daycare facilities, samples were collected from employees, children, and environmental surfaces. Nasal samples were taken from participating children, and nasal and pharyngeal samples were taken from participating employees. All samples were cultured, and *S. aureus* isolates were examined by pulsed-field gel electrophoresis, *spa* typing, and antimicrobial drug susceptibility testing and tested for the Panton-Valentine leukocidin gene. One participant was colonized in the

nose and throat with *t*571, a *spa* type previously reported to correspond to ST398 (*1*). The isolates were nontypeable when *Sma*I was used, also a characteristic of ST398 (*6*). They were digested with *Cfr*9I and found to be closely related to an ST398 isolate of *spa* type *t*034 of swine origin but distinct from *S. aureus* isolated from 2 other employees at the facility (Figure). Both ST398 isolates were susceptible to methicillin.

The colonized employee was a 24-year-old woman who had worked at the facility for ≈5 years. She reported a history of melanoma but was not currently taking any chemotherapy drugs and had not been hospitalized in the previous 12 months. She reported having a family member who worked in a hospital and had direct contact with patients, but the employee lived alone and responded negatively to questions about whether she or immediate family members had had contact with animals or worked in a processing plant.

ST398 may be transmitted from livestock to community members and then from person to person. It can potentially be transmitted in food; several studies have documented ST398 in raw meats (*7*,*8*), and we identified this strain in retail meat products in Iowa (T.C. Smith et al., unpub. data). Secondary transmission of ST398 from colonized persons to contacts has also been suggested, but the few publications reporting this suggest that ST398 seems to be less transmissible by this route than are common human strains (*9*).

We cannot be sure whether either of these routes played a role in acquisition of ST398 by this employee. Although no other tested persons in this childcare facility were found to carry ST398, only 24 (40%) of the 60 employees and 8 (4.8%) of the 168 children participated, suggesting the possibility of a reservoir in the facility among those who were not tested. Of the 24 employees who participated, 2 reported occupational contact with any animals, 2 reported contact with swine, and 3 reported contact with cattle. However, no participant reported having animals other than cats or dogs on their property. It is possible that ≥1 sampled employee may have been a transient ST398 carrier but negative at the time of our sampling.

Reports of ST398 in persons who had no direct contact with livestock in the United States are rare (*10*). To provide a better understanding of the epidemiology of this novel strain, further examination of the emergence of this isolate in community settings and on farms is needed.

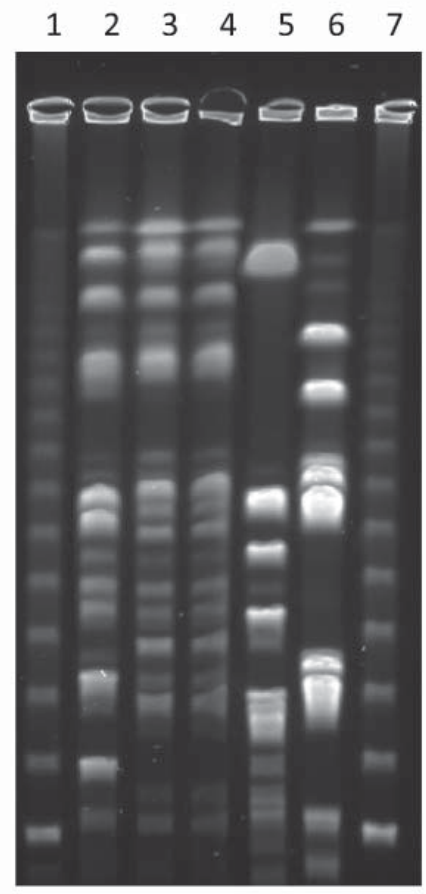

Figure. Pulsed-field gel electrophoresis of *Staphylococcus aureus*. Isolates were digested with *Cfr*9I. Lanes 1 and 7, molecular mass ladder; lane 2, *t*034 sequence type (ST) 398 isolate from pig; lane 3, *t*571 ST398 nasal isolate from colonized childcare employee; lane 4, *t*571 ST398 throat isolate from colonized childcare employee; lanes 5 and 6, non-ST398 isolates (*t*2228 and *t*084, respectively) from 2 other childcare employees.

Acknowledgments

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Sequence Analysis of Feline Coronaviruses and the Circulating Virulent/Avirulent Theory

To the Editor: Feline coronaviruses (FCoVs) occur as 2 pathotypes, feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV). FECV is common in cats, causing mild transient enteritis in kittens, but is asymptomatic in adult cats. In contrast, FIPV occurs sporadically but is lethal. It replicates in monocytes and macrophages and rapidly disseminates throughout the body causing systemic immunopathologic disease (*1–4*).

The relationship between FECV and FIPV has become a matter of debate. Genetic and animal experimental evidence indicates that FIPV arises by mutation from FECV in the intestinal tract of a persistently infected cat; the virus thereby acquires the monocyte or macrophage tropism that enables it to spread systemically and cause FIP (*5–7*,*8*). According to another view, the 2 pathotypes circulate independently in the field. This circulating virulent/avirulent FCoV theory recently was advocated by Brown et al. (*9*). Their conclusion was based on sequence analyses of parts of the viral genome including the matrix (M) gene, phylogenetic analysis of which revealed reciprocal monophyly of the sequences obtained from FIP cases versus those of asymptomatic FECV-infected animals. In addition, the authors suggested 5 aa residues in the M protein to represent potential diagnostic markers for distinguishing virulent FIPV from avirulent FECV (*9*).

To try to verify the findings of Brown et al. (*9*), we determined and analyzed M genes from 43 FCoV genomes, 20 of which came from cats in single-cat households, and 23 from cattery animals. The latter group consisted of 10 asymptomatic healthy cats (FECV; test specimens: feces) and 13 dead cats with FIP confirmed through pathology (FIPV; test specimens: organs, ascites). These animals came from 8 catteries. FECV and FIPV cases were found in 7 (designated A to G); the remaining cattery (H) provided 2 cats with FIP. The genomes from individually living cats were from 15 FIPV- and 5 FECV-infected animals.

Using specific primers (sense 5′-CGTCTCAATCAAGGCATATAATC CCGACGAAG-3′, antisense 5′-CAG TTGACGCGTTGTCCCTGTG-3′), we amplified the same 575-bp M gene fragment as studied by Brown et al. (*9*). GenBank accession numbers for the FCoV M gene sequences determined in this study are HQ738691–HQ738733. When compared by phylogenetic analysis, the nucleotide sequences of FIPV and FECV M genes distributed into paraphyletic patterns rather than in monophyletic clusters (Figure, panel A).

Thus, as we observed earlier for the 3c gene (*10*), M gene sequences generally clustered according to the cattery from where they originated, irrespective of their pathotype (e.g., FECV 586 and FIPVs 584 and 585 from cattery A; FECV 620 and FIPVs 615 and 622 from cattery G; FECV 10 and FIPV 8 from cattery F). Such a distribution pattern is consistent with the mutation theory, according to which FIPVs originate from FECVs and are thus closely related (*7*,*9*). Exceptions in this picture were FIPV 9 in cattery F and FECVs 406 and

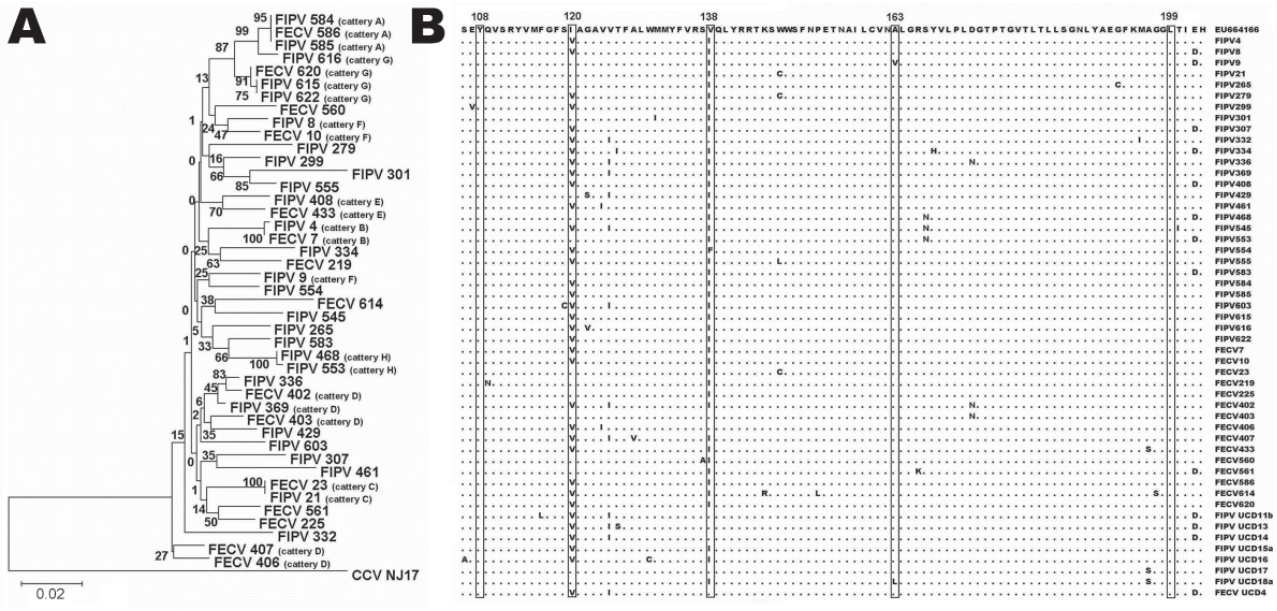

Figure. A) Phylogenetic relationships of feline coronaviruses (FCoVs) detected in feces of healthy cats and in organs/ascites of cats with feline infectious peritonitis. Alignment of the matrix (M) gene sequences was used to generate a rooted neighbor-joining tree with the M gene sequence of canine coronavirus strain NJ17 (Genbank accession no. AY704917) as outgroup. Bootstrap confidence values (percentages of 1,000 replicates) are indicated at the relevant branching points. Branch lengths are drawn to scale; scale bar indicates 0.02 nucleotide substitutions per site. Viruses detected in cattery animals are indicated by a cattery designation after the virus identification number. B) Alignment of amino acid sequences of partial M proteins of the FCoVs from panel A, as compared with a feline infectious peritonitis virus (FIPV) reference sequence (top line) published by Brown et al. (*9*) (GenBank accession no. EU664166), and with 8 American FCoV sequences (bottom) published by Pedersen et al. (*8*). The 5 aa residues at positions 108, 120, 138, 163, and 199, suggested by Brown et al. (*9*) as potential diagnostic sites, are boxed.

407 in cattery D, presumably caused by multiple FCoV lineages in these open catteries (an open cattery is one in which cats can move in and out, usually for breeding purposes).

We also examined the 5 aa sites in the M protein identified by Brown et al. (*9*) as being potentially diagnostic of FIP. An alignment of the relevant part of the polypeptide sequence, comprising the presumed signature residues at positions 108, 120, 138, 163 and 199, is shown in the Figure, panel B, for all FIPV and FECV genomes sequenced in this study. Within this sample collection, we observed complete sequence conservation at positions 108 and 199, virtually complete conservation (1 difference) at position 163. The 2 aa identities (Val and Ile) found at position 120 and 138 occurred with similar frequencies in FIPV and FECV (position 120: Ile in 16/36 [44%] FIPVs and in 6/14 [43%] FECVs; position 138: Ile in 29/36 [81%] FIPVs and in 12/14 [86%] FECVs). These observations do not indicate the slightest tendency of sequence segregation among the 2 pathotypes. In the alignment of the Figure, panel B, we also included M protein sequences translated from several FCoV genomes from the Americas, 7 FIPV, and 1 FECV (*8*). The comparison does not reveal peculiarities indicative of geographic segregation. Hence, our data do not confirm the diagnostic potential of the M protein sequence nor do they support the suggested role of the membrane protein in FIP pathogenesis (*9*).

Informative as it may be, comparative sequence analysis will eventually not be sufficient to answer the FECV/FIPV question. What will be needed is a reverse genetics system to generate and manipulate the FCoV genome as well as a cell culture system to propagate the viruses, both of which have thus far not been achieved.

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Effects of Vaccination against Pandemic (H1N1) 2009 among Japanese Children

To the Editor: We report findings from a household-based study on the protective effects of vaccination against pandemic (H1N1) 2009 among Japanese children. In Japan, prioritized vaccination started in October 2009, focusing on health care workers, pregnant women, persons with underlying diseases, and children 1–9 years of age. Only nonadjuvant split vaccines (inactivated) produced by 4 manufacturers (Denka Seiken, Tokyo, Japan; Kaketsuken, Kumamoto-shi, Japan; Kitasato, Tokyo, Japan; and Biken, Suita-shi, Japan) were used by the end of January 2010 (*1*). Because the protective effects of vaccination at the individual level are best measured by household data (*2*), we conducted a retrospective household survey involving 1,614 nonrandomly sampled households (i.e., based on area sampling of households across Japan, according to the regional population size, with a total of 6,356 household members), in which the earliest cases were diagnosed from October 2009 to mid-February 2010. Our study aimed to assess vaccine-induced reductions in susceptibility and infectiousness among children by using the household secondary attack rate.

Influenza cases were defined as confirmed cases (i.e., diagnosed by real-time PCR) or influenza-like illness (ILI) cases (i.e., in febrile patients [≥37.5°C] with cough and/or sore throat). The cases had to meet the following inclusion criteria for analyses: 1) index case-patient and exposed persons in households were healthy children 1–9 years of age (households with <2 children were excluded), because age-specific susceptibility and infectiousness can greatly influence the frequency of household transmission (*3*–*6*); b) all exposed persons shared the same household with index case-patients for at least 1 of 7 days after illness onset of the index case-patient; c) index case-patient did not receive treatment with antiviral agents (e.g., zanamivir or oseltamivir) within 2 days after illness onset; d) time interval from illness onset of the index case-patient to that of subsequent case-patients was ≤7 days (*7*,*8*); and e) vaccinated persons received their first vaccination >28 days before illness onset (if index case-patient) or exposure (if not index case- patient).

In total, 251 children met the above criteria, comprising 109 index case-patients and 133 unvaccinated and 9 vaccinated exposed persons. The mean age was 6.4 ± 2.1 SD years. Among the 251 children, 15 (6.0%) had been vaccinated, and 169 (67.3%) had received a diagnosis of influenza. Confirmed cases accounted for 17.8% (30/169) of cases; 21 patients were the index case-patients in individual households. The mean age of patients with confirmed diagnoses was 6.5 ± 2.0 SD years and did not differ significantly from the ILI patients.

Let SAR_{ij} represent the household secondary attack rate (SAR) with vaccination statuses of the index patient *j* and exposed persons *i* (where *i* or *j* is 0 or 1 for unvaccinated or vaccinated, respectively), and let *b* represent both groups. Among 133 exposed unvaccinated children, ILI developed in 59, yielding an SAR_{0b} of 44.4%. Among 9 exposed vaccinated children, ILI developed in 1 child, yielding an SAR_{1b} of 11.1%. The difference between these SARs was marginally significant (p = 0.08 by Fisher exact test), and the susceptibility reduction was $1 - SAR_{1b}/SAR_{0b}$ = 75.0% (95% confidence interval [CI] –60.5% to 96.1%). Considering only exposures caused by unvaccinated first patients, SAR_{00} and SAR_{10} were 44.7% (59/132) and 0% (0/4), respectively.

When the first patients with ILI in households were unvaccinated, ILI was observed in 59 of 136 children, yielding an SAR_{b0} of 43.4%. Among 6 exposures caused by vaccinated first patients, ILI developed in 1 person, yielding an SAR_{b1} of 16.7%. Although not significant ($p = 0.40$), the reduction in infectiousness by vaccination was estimated to be $1 - SAR_{b1}/SAR_{b0}$ = 61.6% (95% CI –132.3% to 93.6%). The SAR_{01} was 0% (i.e., 1 exposure to an unvaccinated person caused by a vaccinated first patient did not result in influenza). Limiting the definition of influenza to confirmed cases, all 8 exposures to vaccinated persons did not result in influenza, and SAR_{0b} and SAR_{1b} were 10.8% and 0%, respectively. Similarly, all 5 exposures caused by vaccinated first patients did not result in confirmed cases, and SAR_{b0} and SAR_{b1} were 10.5% and 0%, respectively.

Although the CIs of the estimates included zero because of the small sample size, the expected reductions in susceptibility and infectiousness were 75.0% and 61.6%, respectively, which is consistent with findings from a meta-analysis of vaccine efficacy against seasonal influenza (*9*). Two limitations must be noted, namely, estimates based on nonrandom samples and a case definition that relied on symptoms of case-patients. The former point cannot be explicitly addressed by a retrospective study design, but we enforced strict inclusion criteria for analyses and limited our study to healthy children. Accounting for the latter point (e.g., serologic diagnosis to capture symptomatic and asymptomatic cases) could yield slightly higher estimates than ours, provided that vaccination reduces the probability of clinical illness if infection occurs. Thus, despite these limitations and a critical need for further studies that include estimations of effectiveness (*10*), our results provide insight into the effects of vaccination in reducing risks for infection and clinical attack among children exposed to pandemic (H1N1) 2009 virus in their households.

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Pandemic (H1N1) 2009 Virus in 3 Wildlife Species, San Diego, California, USA

To the Editor: The influenza A pandemic (H1N1) 2009 virus rapidly created a global pandemic among humans and also appears to have strong infectivity for a broad range of animal species (*1–3*). The virus has been found repeatedly in swine and has been detected in a dog, cats, turkeys, and domestic ferrets and in nondomestic animals, including skunks, cheetahs, and giant anteaters (*2–4*). In some cases, animal-to-animal transmission may have occurred, raising concern about the development of new wildlife reservoirs (*2*).

In 2009, the first recognized occurrence of pandemic (H1N1)

2009 in southern California in April was followed by a surge of cases during October through November (*4*). During this time, respiratory illness developed in a 12-year-old male American badger (*Taxidea taxus taxus*), a 19-year-old female Bornean binturong (*Arctictis binturong penicillatus*), and a 7-year-old black-footed ferret (*Mustela nigripes*) housed in a San Diego zoological garden.

The 3 affected animals had clinical signs that included lethargy, inappetance, dyspnea, nasal discharge, and coughing. The severity of disease in the badger and binturong necessitated euthanasia; the ferret recovered with antibiotic and fluid therapy. Postmortem examination revealed bronchopneumonia with diffuse alveolar damage in the badger and interstitial pneumonia with diffuse alveolar damage in the binturong. Bacterial cultures and Gram stains of affected lung samples were negative.

Molecular analyses for several groups of viruses, including *Herpesviridae*, *Paramyxoviridae*, *Adenoviridae*, and all influenza A viruses, were performed on frozen lung samples from the badger and binturong and on frozen conjunctival and pharyngeal swabs from the ferret. Results of PCRs specific for segments of influenza A nucleoprotein, matrix protein, hemagglutinin, and neuraminidase genes were positive in samples from all 3 animals, and DNA sequencing of amplicons identified the viruses as pandemic (H1N1) 2009. Influenza A virus was not detected in samples from the ferret after it recovered. Results of PCRs for all other viruses were negative. Immunohistochemical evaluation of lung samples from the badger for antigens of influenza A virus (*5*) showed rare staining in bronchiolar epithelial cells (Figure).

Respiratory disease in all 3 affected animals seemed to be caused by pandemic (H1N1) 2009 virus. The badger and binturong were generally healthy, no other pathogens were detected, and pulmonary lesions were consistent with influenza pneumonia. In these animals, pandemic (H1N1) 2009 infection was especially aggressive, resulting in irreversible disease. Reports of pandemic (H1N1) 2009 virus in skunks and anteaters also describe severe disease in those species (*2*,*3*).

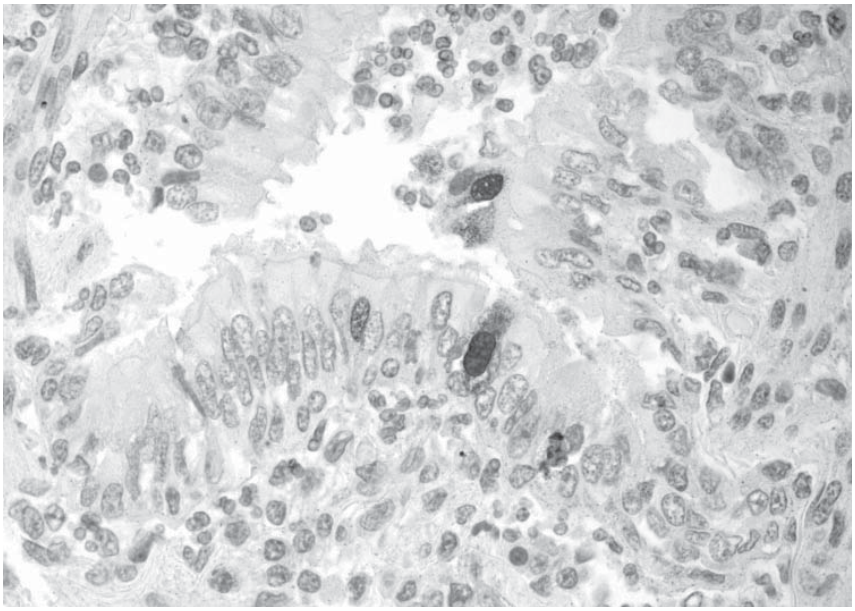

Figure. Lung section from an American badger showing immunohistochemical staining specific for the pandemic (H1N1) 2009 virus within the nucleus and cytoplasm of bronchiolar epithelial cells and concurrent inflammatory cell infiltrates; hematoxylin counterstain. Original magnification ×158. A color version of this figure is available online (www.cdc.gov/EID/content/17/4/747-F.htm).

In contrast, the infected black-footed ferret in our study had relatively mild clinical illness, consisting only of lethargy. This finding was surprising given recent experimental studies that reported the current pandemic (H1N1) 2009 virus was more pathogenic in domestic ferrets (*Mustela putorius furo*) than typical seasonal influenza viruses (*6*). However, several factors could have resulted in the low level of disease in this animal, such as prior immunity to influenza viruses or a low exposure dose. It is also possible black-footed ferrets are innately more resistant to influenza infection than domestic ferrets.

The origin of infection in these cases was not determined but was most likely an infected human. All animals had some level of contact with caretakers or veterinarians and were housed separately from other wildlife species. None of the potential human sources of virus had clinical signs before the animals became ill; however, influenza infections in humans can often be mild (*7*). Wild animals, such as opossums and skunks, that occasionally enter the zoological garden, represent another possible source. Good hygiene and husbandry practices used within the enclosures of the badger, binturong, and ferret failed to prevent infection, which suggests pandemic (H1N1) 2009 is efficiently transmitted to these species. Descriptions of infection in giant anteaters and cheetahs kept under similar conditions also support high transmissibility of influenza A viruses to animals, as do ongoing findings for swine (*3*,*4*,*8*).

Although ferrets are known to be susceptible to influenza A virus, to our knowledge, influenza in badgers and binturongs has not been

reported. Badgers and binturong have been housed in zoological gardens for decades without incidence of influenza. Increased surveillance for influenza by the scientific community during the pandemic may have resulted in the novel recognition of infection in these species. Alternatively, the current pandemic (H1N1) 2009 virus may have a broader host range and stronger virulence than viruses in the past.

Pandemic (H1N1) 2009 was first detected in humans in March 2009 and reached pandemic levels by June of that year, rapidly establishing a rich pool for the development of genetic variants. Naturally acquired disease has now been described in 10 animal species, and experimental infection has been reported in an additional 2 animals (mice and cynomolgus macaques) (*9*). The ubiquity of pandemic (H1N1) 2009 and its ability to infect a diverse range of hosts is worrisome for the health of wildlife and for the possibility of creating additional reservoirs that could alter the evolution of subtype H1N1 viruses by applying varied selection pressures and establishing new ways of generating unique reassortant strains.

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Hemagglutinin 222 Variants in Pandemic (H1N1) 2009 Virus

To the Editor: The biologic role of amino acid variants at position 222 of the hemagglutinin (HA) gene of pandemic (H1N1) 2009 virus in severe infections has been extensively discussed. A recent series of studies (*1–3*) confirm the initial suggestions that G or N in this position might confer greater pathogenic potential to the virus than to the wild type. In contrast, their data suggest that no particular pathogenicity is associated with the 222E variant because it occurs at the same frequency in severe and mild infections. Most authors also seem to agree that D222G or N appears sporadically in phylogenetically distant viruses, with limited transmissibility.

However, Puzelli et al. (*4*) reported transmission of a 222G mutant from son to father (with the appearance of an additional G155E mutation). In Italy, the pattern of D222 variants has been peculiar, with extremely rare appearances of 222G and high diffusion of 222E isolates. At the National Institute for Infectious Diseases in Rome, 82 isolates (GenBank accession nos. CY063455–CY063469 for new sequences in this study) were monitored for D222 variants. No 222G or N variants were detected, even in 24 severe infections, nor was the G155E mutation detected. This finding was not surprising, given the worldwide low frequency of this mutation, even in severe infections.

Conversely, D222E was detected in 12 of the 82 cases, peaking in September 2009, when it was present in most of the infections, with no overrepresentation in severe cases. Subsequently, it was substituted by different 222D viruses during the autumn–winter outbreak. The analysis of publicly available sequences from

other centers in Italy confirmed the trend: 222E was the dominant variant during the summer, 222G was detected only in 4 cases, and 222N was never detected. As we previously reported (*5*), phylogenetic analysis of 222E variants allowed identification of them as an authentic circulating subclade of clade 7 (*6*) or cluster 2 (*7*), rather than as sporadically occurring variants.

To further investigate the origin and the evolution of 222 variants, we have extended the phylogenetic analysis (neighbor-joining) to 2,492 complete HA sequences from the Global Initiative on Sharing All Influenza Data database (expanded Figure online, www.cdc.gov/EID/content/17/4/749-F.htm), confirming the clustering of all the worldwide 222E variants in a well-defined subclade (Figure; expanded Figure online). The same analysis showed that D222G variants could be reconciled with 2 different phylogenetic patterns. The first less frequent pattern includes sequences appearing sparsely throughout the tree, confirming the mentioned hypothesis of sporadic mutation. In contrast, the second pattern (the majority) relates to small groups of sequences appearing in monophyletic microclusters. Among these microclusters, 2 are particularly interesting because they include only 222G sequences, isolated in different parts of the world (expanded Figure online). This finding is still compatible with sporadic mutation; bootstrap values are low because of the low general variability of these sequences. However, the possibility that D222G variants are transmissible and might sustain small epidemics of their own or that they might arise more easily from specific, phylogenetically related backgrounds, is intriguing. In a few countries, such as Italy, Norway, or Sweden, where the 222E virus has been circulating as a substantial proportion of the total virus, the 222G variants appeared more frequently in the genetic context of the 222E virus (*1*,*4*), as demonstrated by phylogenetic analysis and confirmed by the analysis of codon 239 (the codon determining the 222 residue specificity): GAA to GGA (E to G), instead of GAT to GGT (D to G). In these cases, the correct definition of 222G variants would therefore be E222G rather than D222G. From this point of view, the 222N variant would have a higher genetic barrier to change from E because it would require 2 mutations (GAA to AAT) instead of 1 (GAT to AAT), and indeed none of the 16 available (worldwide) 222N full-length variants clustered with the 222E virus. On the basis of these findings, 2 different amino acids, D and E, might be considered polymorphic variants at position 222, and the potentially more pathogenic mutants or circulating variants would be G or N.

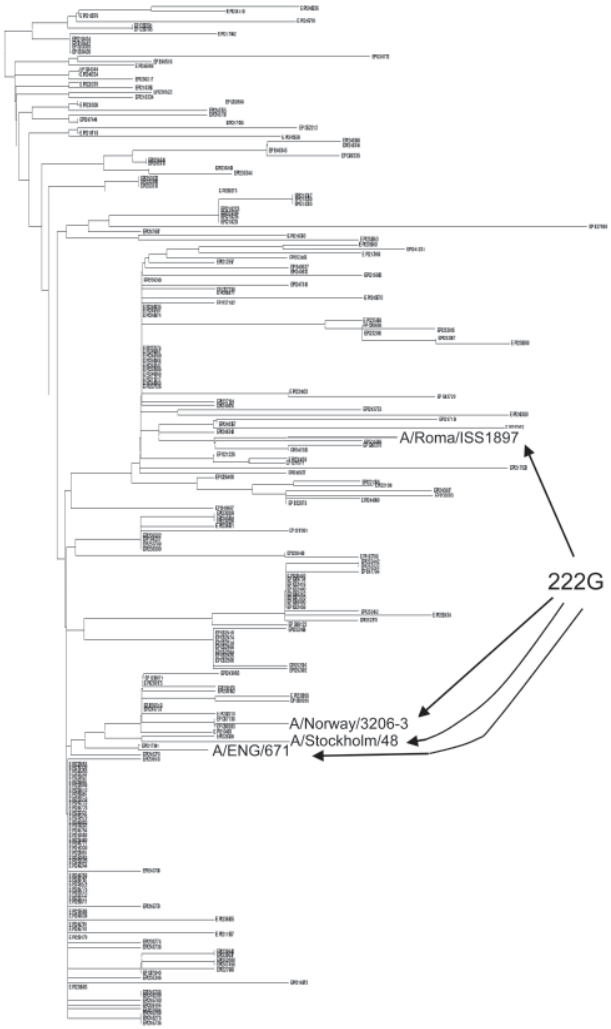

Figure. Monophyletic pandemic (H1N1) 2009 virus D222E cluster, including 98% of the global 222E isolates. E222G variant isolates, as examples, respectively, from Italy (*4*), Norway (*1*), Sweden, and the United Kingdom, are indicated by arrows. The sequence labels represent the Global Initiative on Sharing Avian Influenza Data serial numbers; those of particular interest for this study are indicated by the strain name or country of origin. An expanded, color version of this figure is available online (www.cdc.gov/EID/content/17/4/749-F.htm).

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Effect of School Closure from Pandemic (H1N1) 2009, Chicago, Illinois, USA

To the Editor: On April 28, 2009, the Chicago Department of Public Health received notification of 1 student at an elementary school with a probable pandemic (H1N1) 2009 virus infection; the infection was subsequently laboratory confirmed. This case was one of the first pandemic (H1N1) 2009 cases in Chicago. To prevent transmission of influenza and with guidance from the Chicago Department of Public Health, the school closed on April 29; it reopened on May 6 after the Centers for Disease Control and Prevention (CDC) revised its recommendations (*1*). We conducted an investigation to evaluate psychosocial and economic effects of the school closure on the students' families and to assess whether students complied with mitigation recommendations. In the early pandemic, Chicago's number of pandemic (H1N1) 2009 cases was one of the highest in the United States (*2*).

Households were surveyed if ≥1 child in the household was enrolled in the school and contact was made with an adult (parent/guardian). We made a minimum of 3 attempts to contact eligible households by telephone in English or Spanish. Households without working telephone numbers were visited, but only 1 visit yielded a completed interview. The school had an enrollment of 744 students (609 households, of which 439 were reachable by telephone) during April–May 2009. The final sample comprised 170 households (39% of reachable households). Fifty-four (31%) respondents were employed full-time and 37 (22%) part-time; 78 (46%) were unemployed, homemakers, students, or retired. Households had a median of 2 adults and 2 children in grades prekindergarten through eight.

In contrast with findings of Johnson et al. (*3*) in an investigation of an influenza B virus outbreak, where 89% of students visited ≥1 public location during the school closure, results from our investigation (Table) indicate that most students complied with recommended social distancing measures. Johnson et al. highlighted the potential for transmission in public areas during a school closure. However, with only approximately one third of households in this investigation reporting their children went to public areas during the school closure, the same level of concern of public transmission was not found.

The results from this investigation indicate the economic effect of the school closure was minimal for survey respondents. These results were similar to those found by Johnson et al. (*3*), which had only 18% from 220 households (with 315 employed adults) report missing work to stay home because of school closure. However, the number of families losing work time in our investigation was much lower than the 53% of families in central Virginia reported by Nettleman et al. (*4*) using a survey of school absenteeism and employment status for adults who stayed home to care for an ill child. This might have been because 31% of respondents surveyed in this investigation were homemakers, and an additional 10% were unemployed or retired. Therefore, many parents and legal guardians from this investigation did not need to noticeably change their daily routine to care for their children during the closure. Moreover, compliance has been shown to vary by income and employment status (*5*).

CDC guidance issued on April 27, 2009, recommended closing any school that had a laboratory-confirmed case of pandemic (H1N1) 2009 (*1*). As new information became available, CDC updated its recommendations,

reflecting consideration of the overall benefits and harms, including students being left home alone, parents missing work to care for their children, students missing meals, and students' education being interrupted (*1*). The findings from investigating the effect of this school closure support other CDC recommendations and are relevant for future pandemics.

Our study was limited by the low household participation rate, which might have biased the current findings. However, student characteristics, including race/ethnicity, grade level, and enrollment in free/reduced lunch and special education services received, were consistent with demographics of the school (*6*).

This relatively brief school closure had limited effect on the families in our study, but a school closure in a different community, at a different time, or perhaps of longer duration than 1 week might have a greater effect and prove to be more difficult for parents. The public health benefits of future school closure might increase if strategies were implemented to increase students' compliance with recommendations to avoid public places or group gatherings to decrease exposure to pandemic (H1N1) 2009 and seasonal influenza. In addition, parent education on infection control strategies is necessary to increase compliance. However, strategies should limit the disruption to day-to-day activities of families and learning in the schools. Interruptions in school lunch programs might be offset by providing meals in noncongregate settings outside of school or involving community organizations. Further research is needed to understand the economic effect and timing of school closures in other populations or communities, and to understand the efficacy of school closure on reducing transmission of other communicable diseases.

Table. Household responses (n = 172) related to school closure as a result of pandemic (H1N1) 2009, Chicago, Illinois, USA, April 29–May 5, 2009

Response	No. (%)
Highest education level of parent or guardian	
None	2 (1)
Elementary school	48 (28)
Junior high school	9 (5)
High school	59 (34)
Some college	29 (17)
Advanced degree	21 (12)
No response	4 (2)
Employment status of parent or guardian	
Full time	54 (31)
Part time	37 (22)
Student	6 (4)
Retired	2 (1)
Unemployed	17 (10)
Stay-at-home	53 (31)
Other/no response	3 (2)
Receipt of closure information by parent or guardian*	142 (84)
School	89 (63)
Radio or television news	81 (57)
Other parents/students	17 (12)
Student	5 (4)
Press conference	4 (3)
Internet	3 (2)
Found closure difficult for self or family*	105 (61)
Fear about H1N1	74 (70)
Uncertainty about duration of closure	70 (66)
Fear about family's health	66 (62)
Schedule changes	33 (31)
Student missing school meals	26 (25)
Child care arrangements	21 (20)
Loss of income because of lost work time	17 (16)
High cost of child care arrangements	13 (12)
Transportation difficulties	12 (11)
Student missing education	3 (3)
Behavioral concerns related to disability	1 (1)
Student activities during closure*	
Did homework	125 (73)
Went to a public place	63 (37)
Went to home of another family member	43 (25)
Got together with <6 friends	29 (17)
Went to afterschool extracurricular activity	20 (12)
Got together with >6 friends	13 (8)
Slept at a friend's house	5 (3)
Went to afterschool program	5 (3)
Alternate child care arrangements made†	13 (8)

*Response categories were not mutually exclusive.
†Mean cost of alternate childcare $45.

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Imported Rabies, European Union and Switzerland, 2001–2010

To the Editor: Europe is progressively becoming free of sylvatic rabies. However, reintroduction remains a threat. We report the incidence of rabies importation into the European Union (EU) and Switzerland and highlight common pathways for rabies introduction.

Rabies is a notifiable disease within the EU. Through comprehensive oral vaccination campaigns most EU member states have eliminated the disease. Despite this success, the danger of reintroduction of the disease is ever present. Rabies could be reintroduced through direct reentry of infected animals across land borders, as has happened in eastern Italy with an outbreak of fox rabies originating from the western Balkan region (*1*). Alternatively, reintroduction can occur through illegal or accidental import of an infected animal.

In an attempt to mitigate direct reentry, vaccination campaigns and technical assistance in nonmember states and in the EU have attempted to reduce the incidence of disease throughout the continent. Avoidance of importation of infected companion animals is achieved through enforcement of legislation. EU regulation No. 998/2003 defines the requirements that dogs or cats must meet before entry into the EU with the aim of preventing an infected but asymptomatic dog or cat from entering a member state from another country. Entry requirements include that the animal is identifiable by a microchip or tattoo, has been vaccinated against rabies, and, depending on the status of the country of origin according to Annex II of regulation No. 998/2003, has been serologically tested. A veterinary certificate should accompany the animal during the period of travel. Failure to meet these requirements could lead to the animal being returned to its country of origin, isolation of the animal until it meets the requirements, or, as a last resort if the first 2 options are not feasible, euthanasia of the animal. Certain member states also stipulate application of antiparasite treatments before entry.

Despite these regulations, importation of animals incubating rabies can still occur through failure of border controls, ignorance of importation rules, or active subversion of these rules. The online Technical Appendix (www.cdc.gov/EID/content/17/4/753-Techapp.pdf) lists documented cases during 2001–2010 of rabid dogs brought into the EU. Control measures in the form of euthanizing animals and contact investigation have ensured that the disease did not become established in a carnivore reservoir and no human incidence of disease resulted. Vigilance at the level of veterinary practitioners has also enabled quick discovery of suspected animal cases, which limited the number of secondary animal cases (online Technical Appendix). However, these discoveries point to a persistent trend of illegal animal movement into the EU.

Ignorance on the part of tourists of the danger of importing infectious disease and the rules governing animal movement underpin most of the cases. Juvenile dogs feature in many of the reports, presumably because puppies are attractive to tourists and, being small, are easily moved (*2*,*3*). A recurring pathway is that of vacationers visiting Morocco and returning to France through the Iberian Peninsula. This pathway has been confirmed in 4 cases and is suspected in several other instances (online Technical Appendix; *4*). In addition, rabies in dogs has been reported in the Spanish enclaves of Ceuta and Melilla on the north coast of Africa in recent years (*5*). A further route of introduction appears to be

from western Balkan countries into Germany (*6*).

The costs associated with such introductions are numerous. These costs include the diagnostic investigation of suspected cases, particularly if molecular analysis is required to confirm the source of the incursion, as was required when rabies was detected in a puppy in Switzerland (*7*).

Subsequent investigations to identify animal and human contact cases, often requiring >1 national or international agency, are needed to ensure that the disease has not spread and potential human contacts receive appropriate postexposure prophylaxis. In some cases the implementation of hotlines and several press releases was necessary to cope with the demand for information by the public (*4*). However, although media attention in such cases reached its primary and immediate objective, i.e., no secondary human rabies cases were reported, it may also have contributed to enhancing the sense of rabies risk, thereby prompting persons to associate dog bites in general with rabies and thus leading to increased numbers of persons seeking postexposure prophylaxis unnecessarily for several months (*8*). Further costs are also incurred in the euthanasia or quarantine of contact animals.

The evidence suggests that this trend for importation of animals incubating rabies will continue, requiring member states to maintain vigilance with measures appropriate to the potential risk and consequences of a rabies outbreak. This vigilance should involve rapid investigation of suspected cases of disease, maintenance of rabies diagnostic capacity and contingency plans, and improved coordination between member states to deal with disease introduction.

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Cytomegalovirus Viremia, Pneumonitis, and Tocilizumab Therapy

To the Editor: Tocilizumab is a monoclonal antibody that competitively inhibits binding of interleukin-6 (IL-6) to its receptor. It is approved for treatment of rheumatoid arthritis (RA) as monotherapy or with methotrexate. We report a case of cytomegalovirus (CMV) disease complicating treatment with an IL-6 receptor antagonist.

A 41-year-old man who had a diagnosis of nonerosive RA (seronegative for rheumatoid factor and anticyclic citrillinated peptide antibody) in 1994 had fevers in May 2010. Previous treatment included etanercept, methotrexate, and various doses of prednisone (highest dose 40 mg/day). Because of uncontrolled RA, he was treated with monthly infusions of tocilizumab, 600 mg (≈4 mg/kg, first infusion in March 2010 and the second in April 2010), methotrexate (7.5 mg/week), and prednisone (5 mg/day from April 2010 onwards).

Fever, a productive cough with white sputum, and wheezing developed ≈3 weeks after his second infusion of tocilizumab, which resulted in RA symptom resolution (Figure). Tapering of steroid treatment and levofloxacin resulted in some improvement. However, after 1 week, persistent fever led to hospitalization. Worsening shortness of breath, nausea, and vomiting developed. Results of computed tomography (CT) scans of the chest, abdomen, and pelvis were unremarkable. He was transferred to the Cleveland Clinic because of hypotension and intravenous dye–induced renal failure.

Daily fever (≤103°F), shortness of breath, nausea, and mild diarrhea persisted. After cultures were obtained, he received 1 g vancomycin,

3.375 g piperacillin/tazobactam, and 5 mg/kg lipid amphotericin B (empiric therapy). After a single dose of these drugs, antimicrobial drugs were withheld. Methotrexate and tocilizumab were also withheld. Prednisone (5 mg/day for his duration in the hospital and after discharge) was continued and resulted in resolution of RA symptoms.

Laboratory testing (reference ranges) showed leukocyte count 1,850 cells/μL, hematocrit 26.8%, platelet count 21,000 cells/μL, aspartate aminotransferase 107 U/L (7–40 U/L) alanine aminotransferase 56 U/L (5–50 U/L), alkaline phosphatase 164 U/L (40–150 U/L), and serum creatinine 3.15 mg/dL (0.7–1.4 mg/dL). Testing included negative serologic results for *Bartonella* species, *Coxiella burnetii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, hepatitis A, B, and C viruses, HIV, and parvovirus B19; negative PCR results for *H. capsulatum* and *Legionella pneumophila* antigens, influenza A/B viruses, respiratory syncytial virus, and human herpesvirus 6; no growth for routine fungal and mycobacterial blood cultures, urine cultures, and stool cultures; negative direct immunofluorescence results for adenovirus, parainfluenza viruses, and human metapneumovirus; and negative stool results for ova and parasites. A bone marrow biopsy did not show any abnormalities.

PCR showed CMV viremia (maximum value 50,413 copies/mL whole blood). A test result for immunoglobulin G against CMV was positive, indicating reactivation of latent infection. Epstein-Barr virus (EBV) viremia was low (1,821 copies/mL whole blood). Although the patient likely showed clinically irrelevant EBV shedding, fatal reactivation of EBV during tocilizumab therapy has been reported (*1*).

Results of CT scans of chest, sinuses, abdomen, and pelvis on admission at our institution were unremarkable. However, scanning of indium 111–labeled leukocytes 12 days after admission showed bilateral pneumonitis, and repeat chest CT showed interval development of ground glass opacities in the right upper lobe.

The patient was treated with intravenous ganciclovir for 10 days at doses adjusted for renal failure. Treatment was changed to oral valganciclovir, 900 mg 2×/d for 20 days, upon discharge. His symptoms gradually improved, and he had no fever after ≈7 days of treatment. His CMV DNA level decreased to 4,996 copies/mL after 3 days of therapy. A negative result for CMV DNA was observed 14 days after starting therapy. Thirty-five days after starting therapy, a CMV DNA test result remained negative, leukocyte count increased to 3,740 cells/μL, hematocrit to 32.3%, and platelet count to 194,000 cells/μL. These findings suggest that pancytopenia was likely secondary to CMV infection. Creatinine level returned to the reference range, and liver enzyme levels improved (aspartate aminotransferase 52 U/L, alanine aminotransferase 73 U/L). Cytopenia and liver toxicity are side effects of treatment with tocilizumab (*2*). His condition showed improvement at follow-up 35 days after starting therapy. He continued to receive prednisone (5 mg/day) and RA symptoms were controlled.

Similar to therapeutic blockade of tumor necrosis factor-α (TNF-α), tocilizumab has been associated with increased risk for infections. Several cases of CMV disease complicating TNF-α blockade, including pneumonia, have been reported (*3*). As in this patient, the effect of steroids on risk for infection often cannot be determined. Given the role of IL-6 in antiviral immunity, CMV reactivation in IL-6 blockade is not surprising (*4*). Frequent adverse events are upper respiratory tract infections, headache, nasopharyngitis, and gastrointestinal symptoms (*4*). Rates of serious infections were 5.3 infections/100 patient-years in placebo-treated patients and 3.9 infections/100 patient-years in patients treated with tocilizumab for 6 months (*2*). This rate was 7.2 infections/100 patient-years after 3 months of TNF-α

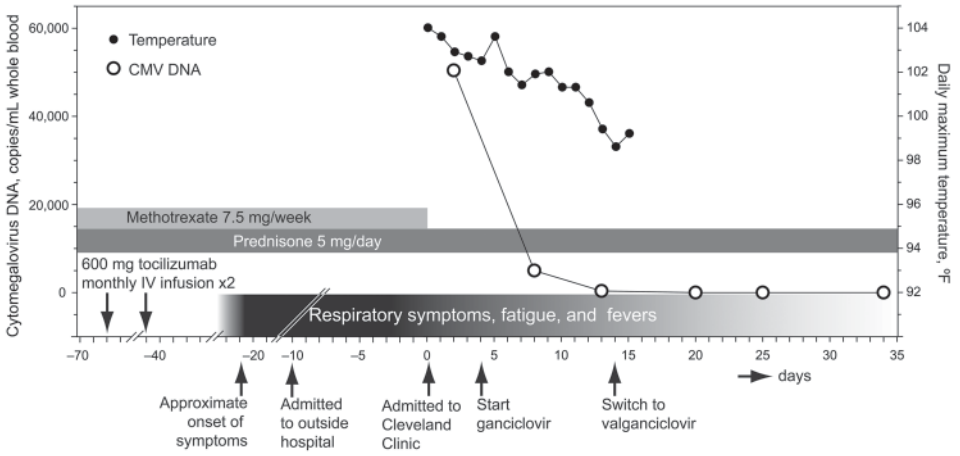

Figure. Timeline of events for a 41-year-old man with rheumatoid arthritis. CMV, cytomegalovirus; IV, intravenous.

blockade (*5*). Other opportunistic infections that have been reported in clinical trials include *Pneumocystis jirovecii* pneumonia, herpes zoster, EBV hepatitis, tuberculosis, and asymptomatic *Mycobacterium avium–intracellulare* (*6*–*10*). Thus, CMV disease should be considered when patients receiving tocilizumab have febrile syndromes.

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Concurrent Influenza and Shigellosis Outbreaks, Papua New Guinea, 2009

To the Editor: A high case-fatality ratio has often been associated with outbreaks of a new influenza virus but is less commonly reported in association with seasonal influenza. Nevertheless, in developing countries, seasonal influenza has been associated with a high proportion of deaths, especially among remote populations. In Madagascar, seasonal influenza mortality rates of 2.5% have been reported (*1*), with even higher rates (15%) reported in Indonesia (*2*) and in the highlands of Papua New Guinea (9.5%) (*3*). High mortality rates during influenza outbreaks in the developing setting have been ascribed to a lack of access to antimicrobial drugs to treat cases of secondary pneumonia and lack of access to health care in general (*1*).

Diarrheal disease is a major cause of illness and death throughout the world, with diarrheal outbreaks causing a substantial proportion of deaths (*4*). Endemic shigellosis is responsible for ≈10% of all cases of diarrhea among children <5 years of age living in developing countries and up to 75% of diarrheal deaths (*5*,*6*). Although epidemic *Shigella dysenteriae* causes the most dramatic form of *Shigella* spp. infections in developing countries with high attack rates and mortality rates, approximately half of the *Shigella* spp. infections are caused by endemic *Shigella* spp. (*4*). Despite the endemicity of both influenza viruses and *Shigella* spp. in developing countries, data on their co-infection are lacking.

In mid-August 2009, an outbreak of bloody diarrhea and influenza-like illness (ILI) was reported to health authorities in Menyamya, a remote highland region of Morobe Province, with an estimated population of 10,000 persons. On August 28, an investigation was conducted to identify the cause and extent and to implement control measures.

Two sets of data were collected at the Hakwange Aid Post in Menyamya: 1) laboratory-investigated cases, 2) verbal autopsies. An additional dataset of clinical cases was subsequently collected from surrounding facilities in the district.

Rapid verbal autopsies were conducted by using standardized questionnaires. Bloody diarrhea was

defined as acute onset of fever and diarrhea with visible blood in the stool. ILI was defined as acute onset of fever with cough or sore throat or both. Twenty deaths were identified in the Hakwange Aid Post catchment area, of which 11 were associated with bloody diarrhea and 9 with respiratory illness. Molecular methods were used to identify and characterize respiratory pathogens, and sequencing was used to identify genes that conferred enhanced pathogenicity. Influenza A virus was identified in 14 of 20 respiratory samples collected, of which 10 were subtyped as H3N2; the virus was A/Perth/16/09-like. During the investigation, patients with ILI were given oseltamivir.

Rectal swab specimens were transported in Cary-Blair media and were cultured within hours before serologic and biochemical testing were performed. Antimicrobial drug resistance testing was performed by using the Kirby-Bauer method. *S. flexneri* serotype 3 was isolated in 3 of 14 investigated cases of bloody diarrhea, with no other pathogens identified. *Shigella* spp. were resistant to amoxicillin, chloramphenicol, and co-trimoxazole but susceptible to ciprofloxacin. Patients received co-trimoxazole and, following sensitivity test results, ciprofloxacin or norfloxacin. Community health education sessions were conducted, and soap, jerry cans, and Aquatabs (Medentech Ltd, Wexford, Ireland) were distributed to households.

Early detection and intervention in disease outbreaks enable timely public health measures and may limit illness and death (*7*). Twenty deaths had already occurred in this provincial border community before our assessment, and an additional 200 deaths were associated with these conditions in neighboring provinces (*8*). The delayed reporting of these events from extremely isolated areas resulted in a delayed and less effective response. Although dealing with an outbreak is extremely challenging in this setting, strengthening the system for reporting such events from the district level has the potential to save lives.

Despite the high number of deaths associated with this outbreak of seasonal influenza A (H3), phylogenic analysis showed that the strain was similar to the low pathogenicity seasonal influenza virus that had circulated in the region during the previous 12 months. In our assessment, only 29% of those who sought treatment for respiratory symptoms and difficulty breathing were given antimicrobial drugs. The facility-based case-fatality ratios suggested a greater likelihood of death associated with possible co-infection (odds ratio 2.1, 95% confidence interval 0.5–7.4) (Table), but the difference was not significant. The major limitation of this investigation is the lack of microbiologic confirmation to allow wider assumptions to be made about possible co-infections, their effects (if any), and the role of other pathogens that cause similar clinical features.

Ciprofloxacin is now recommended as the drug of choice for all patients with bloody diarrhea, regardless of their age (*9*). *Shigella* spp. have widespread resistance to the recommended treatment for bloody diarrhea in Papua New Guinea, co-trimoxazole, and no resistance to ciprofloxacin. This outbreak strain was resistant to co-trimoxazole, and its administration would have contributed little to limiting disease and its subsequent transmission. In the context of widespread illness

Table. Descriptive epidemiology of concurrent outbreaks of bloody diarrhea and influenza-like illness, Menyamya District, Papua New Guinea, 2009

Variable	No. (%) patients*				
	Bloody diarrhea, n = 50	Influenza-like illness, n = 431	Possible co-infection, n = 131	Nonfebrile respiratory illness, n = 92	Total, n = 704
Aid post					
Hakwange	25 (50.0)	256 (59.4)	50 (38.2)	67 (72.8)	398 (56.5)
Kome	10 (20.0)	64 (14.9)	27 (20.6)	12 (13.0)	113 (16.1)
Kulolonguli	15 (30.0)	111 (25.8)	54 (41.2)	13 (14.1)	193 (27.4)
Male sex	24 (48.0)	206 (47.8)	71 (54.2)	40 (43.5)	341 (48.4)
Age group, y					
<5	11 (22.0)	118 (27.4)	33 (25.2)	10 (10.9)	172 (24.4)
5–14	14 (28.0)	103 (23.9)	23 (17.6)	22 (23.9)	162 (23.0)
15–44	21 (42.0)	169 (39.2)	58 (44.3)	50 (54.4)	298 (42.3)
≥45	4 (8.0)	39 (9.1)	17 (13.0)	8 (8.7)	68 (9.7)
Unknown	0	2 (0.5)	0	2 (2.2)	4 (0.6)
Date of onset					
June	0	6 (1.4)	3 (2.3)	2 (2.2)	11 (1.6)
July	9 (18.0)	36 (8.4)	21 (16.0)	7 (7.6)	73 (10.4)
August	29 (58.0)	294 (68.2)	76 (58.0)	57 (62.0)	456 (64.8)
Unknown	12 (24.0)	95 (22.0)	31 (23.7)	26 (28.3)	164 (23.3)
Died	1 (2.0)	8 (1.9)	5 (3.8)	2 (2.2)	16 (2.3)

*Categories are mutually exclusive.

and death, possibly associated with multidrug-resistant *Shigella* spp., a review of the national policy for the management of bloody diarrhea is urgently needed.

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Vol. 16, No. 12

An online Technical Appendix was omitted from the article *Mycobacterium tuberculosis* Infection of Domesticated Asian Elephants, Thailand (T. Angkawanish, et al.). The article has been corrected online (http://www.cdc.gov/eid/content/16/12/1949.htm).

In Memoriam: Frank John Fenner (1914–2010)

Frederick A. Murphy

Frank John Fenner (Figure), one of the world's most distinguished virologists and a dear friend of many colleagues around the world, died in Canberra, Australia, on November 22, 2010, at the age of 95. This In Memoriam must be different from those usually published here. After all, quite detailed pieces are anticipated from the Australian Academy of Sciences, the Royal Society, et al., and Frank had published a comprehensive autobiography (*1*). Thus, there is opportunity to present personal memories, hoping to provide more of a sense of the man, the colleague, and the friend of so many members of the global virology community. This tribute seems to fit in with the closing paragraphs of Frank's autobiography, in which he reflects on friendship and special friends.

Frank was born in Ballarat, Victoria, Australia, but his family moved to Adelaide, South Australia, when he was 2 years old. He received a bachelor of medicine and surgery degree (1938) and a doctor of medicine degree (1942) at the University of Adelaide. During 1940–1946, he was an officer (Captain, Major) in the Australian Army Medical Corps and served in Australia, Palestine, Egypt, New Guinea, and Borneo. At various times, he worked as a medical officer in a field ambulance and casualty clearing station, a pathologist in a general hospital, and, most prominently, as a malariologist. For his work in combating malaria in Papua New Guinea, he was made Member of The Order of the British Empire. As Frank noted later, the highlight of his military service was meeting and marrying a nurse, his wonderful wife, Ellen, known to all as Bobbie. Bobbie died in 1995, but ever after Frank said that his marriage was the most important part of his life. Frank is survived by his daughter, Marilyn, grandson, Simon, granddaughter Sally, and great-grandson Jasper.

After his war-time service, Frank was recruited to work at The Walter and Eliza Hall Institute of Medical Research in Melbourne by Sir Frank Macfarlane Burnet. Initially, they worked on ectromelia virus infection in mice (to explain his work, Frank coined the term mousepox). In 1949, he received a fellowship to study at the Rockefeller Institute for Medical Research in New York City, where he worked in the laboratory of René Dubos. While Dubos worked on *Mycobacterium tuberculosis*, Frank worked on *M. ulcerans*, the etiologic agent of Buruli ulcer, the third most common mycobacterial disease worldwide after tuberculosis and leprosy.

Figure. Frank Fenner at the John Curtin School of Medical Research, Canberra, Australia, inoculating embryonating eggs with myxoma virus, 1950.

Used with permission of the John Curtin School of Medical Research.

Returning to Australia in 1949, Frank was appointed Professor of Microbiology at the new John Curtin School of Medical Research (JCSMR), a unit of the Australian National University (ANU), in Canberra. He then began studying myxoma virus infection in rabbits. Throughout the 1940s and 1950s, Australia had severe rabbit plagues. Frank's classic work on myxomatosis culminated in his classic 1948 paper (*2*). This paper includes a description of the progression of mousepox infection in mice, from the time/site of virus entry, to the time/sites of infection of major target organs, to the time/sites essential for virus transmission. It is still featured in all virology texts, and it marks the beginning of modern research in viral pathogenesis. At the same time, this work provided the scientific basis for release of myxoma virus for biologic control of rabbits. This work became the foundation for understanding the parallel evolution of the virus toward lesser virulence and the evolution of the rabbit toward more resistance to infection; it is another classic concept featured in virology texts.

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Frank became director of the JCSMR in 1967 and served in this role until 1973. He made major changes in the school across broad areas of biomedical science, developing and changing the departments of genetics, medical chemistry, clinical science, human biology, immunology, and pharmacology, and guided the microbiology, immunology, and virology faculties toward world prominence. Imagine one building housing (not all at the same time) such greats as Gordon Ada, Alan Bellett, Bob Blanden, Stephen Boyden, John Cairns, Peter Cooper, Colin Courtice, Peter Doherty, Jack Eccles, Stephen Fazekas de St. Groth, Adrian Gibbs, Alfred Gottschalk, Royal Hawkes, Dick Johnson, Bill Joklik, Kevin Lafferty, Graeme Laver, Fritz Lehmann-Grube, George Mackaness, Barrie Marmion, Ian Marshall, Brian McAuslan, Peter McCullagh, Cedric Mims, Bede Morris, Chris Parrish, Ian Ramshaw, Rob Webster, David White, and Gwen Woodroofe.

And on the side, Frank published 23 books, of which 4 have become symbols of the march of virology through one of its most expansive eras: The Biology of Animal Viruses (2 editions) (*3*), written with Brian McAuslan, Joe Sambrook, David White, and Cedric Mims; Myxomatosis (recently republished) (*4*), written with Francis Ratcliffe; Medical Virology (4 editions) (*5*), written with David White; and Veterinary Virology (3 editions) (*6*), written with Paul Gibbs, Michael Studdert, Peter Bachmann, David White, Rudi Rott, Marian Horzinek, and Fred Murphy. All of Frank's books are incredibly readable; he cited the influence of his father in developing his writing style: "Always generalize." Somehow, in books so full of hard experimental data and proven facts, he always seamlessly added his magic, his interpretative skills, and his clear use of the King's English.

In 1977, the World Health Organization (WHO) named Frank the chairman of the Global Commission for the Certification of Smallpox Eradication. The last known case of naturally transmitted smallpox occurred in Somalia in 1977, and WHO set in place a comprehensive global program to make sure that pockets of infection had not been overlooked. Eradication of the disease has been regarded as one of the greatest achievements in human history, and it was Frank's great honor to officially pronounce global eradication to the World Health Assembly in May 1980. He later said that making this pronouncement stood out most among his many achievements: "[The pronouncement] was accepted unanimously by the World Health Assembly on that day."

Frank had an abiding interest in the environment, and in 1973 was appointed foundation director of the Centre for Resources and Environmental Studies at ANU; he held this position until his retirement in 1979. The Centre became part of the Fenner School of Environment and Society in 2007. It was in this role, late in his career, that Frank publicly expressed a gloomy prospect for the future of humankind: environmental degradation, global warming, overpopulation. In retrospect, this view now seems at odds with Frank's otherwise positive view of life, especially in regard to the grand prospects of advances in medical science in support of human welfare.

Frank played many national and international leadership roles (*1*), but 2 in particular were of great interest to him and central in the advance of science in Australia and virology internationally. The first role was in the Australian Academy of Sciences. Frank was in the first group of scientists to become fellows of the Australian Academy of Sciences, and over the years served in several roles. He served as secretary for biologic sciences, where he spearheaded studies of fauna in Australia and became a leader and financial supporter of environmental conservation programs. Later, this grew into the Fenner Conferences on the Environment and the Fenner Medal for Plant and Animal Sciences.

The second role was in the International Committee on the Taxonomy of Viruses. Frank had been interested in virus taxonomy from his first days working in the field. He became a charter member of the International Committee on Nomenclature of Viruses (changed to the International Committee on the Taxonomy of Viruses in 1973) as it was established by Sir Christopher Andrewes, André Lwoff, and Peter Wildy at the International Congress of Microbiology in Moscow in 1966. Frank was unable to attend the next Congress in Mexico City in 1970, but in his absence was elected the second president of the committee (the first president was Peter Wildy). These were formative days; the basic structure of virus taxonomy as we know it today emerged from the work of the committee, under the presidencies of Peter Wildy and Frank Fenner, as the committee met at the first 3 International Congresses of Virology (Helsinki, 1968; Budapest, 1971; and Madrid, 1974) (*7*).

Over the years Frank received many honors (*1*). However, some are listed to illustrate his preeminent place in the world of science, virology, and public service: Companion of the Order of St. Michael and St. George, Companion of the Order of Australia, Foreign Associate of the US National Academy of Sciences, the Australian Prime Minister's Prize for Science, the Japan Prize, the Copley Medal of the Royal Society, the WHO Medal, and the Albert Einstein World Award for Science. Frank was also proud that the Frank Fenner Building, which houses the ANU Medical School and Faculty of Science, and a residential college, Fenner Hall, are named in his honor.

Turning to a more personal viewpoint, I was fortunate to spend 1970–1971 at the JCSMR as an honorary fellow in the laboratory of Cedric Mims. At the time, Frank was director of the School. It was an amazing time, with

the outstanding virology faculty well in place, most having been recruited by Frank in preceding years. It was an institution where conceptualization preceded experimentation, that is, where everyone thought a lot about the anticipated results and meaning of his or her experimental work beforehand.

One of the keys to the intellectual energy level was morning coffee, a lost art. At ≈10:30 AM, small groups formed in the departmental lounge, and discussion began, often leading to cryptic notes written on napkins, in some instances leading to collaborative papers in the Journal of Experimental Medicine, the Journal of Infectious Diseases, or such. On occasion, small meetings were held in Frank's office, in some instances with guests, with collaborative projects involving virologists from other institutions and other countries following. I recall one such meeting in Frank's office where the director of the Commonwealth Scientific and Industrial Research Organisation had dropped by to bring up a new wrinkle in the use of myxoma virus for rabbit control across Australia. In all this discussion, there was an infectious verve, a sense that the edges of the virologic and immunologic sciences were about to be breached, again. Upon returning to the Centers for Disease Control, I tried to transplant this scientific lifestyle, and although successful within our small unit, I realized that by achieving such a productive and satisfying lifestyle across the entirety of the JCSMR, Frank had again used his magic.

Later, another of Frank's characteristics was made clear to me: his prodigious capacity for work and for the highest ethical standard in dealing with data, his colleagues, and his students. He wrote (*1*), "From childhood, I have been an early riser… getting up when I woke at about 5 AM… usually arriving at the School between 6 and 7 AM." I recall that Frank's car was always in the first parking space; if it was not, it meant he was out of town. This work ethic extended to the writing/editing of various editions of the books Veterinary Virology (*6*) and Medical Virology (*5*). Frank would come to Atlanta for a week at a time and stay at our house. With thousands of marginal notes made beforehand, we would spend endless hours going over chapters. Usually I thought the effect was chaotic, but then a few weeks later I would be sent the most lucid, organized drafts, all in Frank's uniquely smooth, clear, expansive style. I recall times when my wife would call that dinner was ready and I would crumple in anticipation of a drink and a bit of a rest, only to be rejoined after a wonderful dinner and family conversation by Frank's gentle voice, "time to get back to work." I recall that in all this writing/editing, Frank held to a high level of objectivity and honesty in the prose; this was rather like the concept of "evidence-based medicine" of today. I attribute this skill to Frank's sense that the written word must reflect an underlying ethical standard, one that we all should emulate.

I could go on, but perhaps there is place for one more personal memory (I hope my memories prompt others to recall their own). This memory can start with Frank's vignette in his autobiography (*1*): "Bozeman and Yellowstone National Park, 11–25 July 1997: I had been invited to give the Edwin H. Lennette Memorial Lecture at the annual meeting of the American Society for Virology, in Bozeman, Montana, in July 1997. Fred Murphy got in touch with me well before the meeting and suggested that I accompany his family on a week's trip through Yellowstone and Grand Teton National Parks. Fred had an RV (recreational vehicle), which had beds, shower, toilet, stove, and refrigerator. Fred, Irene, son Rick and his 2 young boys, son Tim and his wife, son Terence and his wife, and I travelled from Bozeman and throughout the parks in the RV; his sons had also brought their bicycles. We had a wonderful trip all around Yellowstone, which was not only the first national park in the USA, but one of the most wonderful in the world, with entrancing hot springs, geysers and waterfalls. We also had a great float trip down the Snake River beneath the Tetons. Then we went to Bozeman, where the meeting was very interesting. I gave my lecture and saw a lot of old friends, including Joe Esposito, Grant McFadden, Olin Kew, Dick Moyer and Mary Estes."

This trip was one of the most memorable camping trips the Murphy family had ever taken, and Frank became family immediately. I recall seeing Frank walking down a wide trail with 2 of my daughters-in-law, 1 on each arm, all chatting and laughing; I have always wondered what they were talking about. I recall finding a bottle of scotch in a cupboard of the RV, which Frank and I used pharmaceutically at the end of each day of adventure as we sat before dinner in the Murphy perfectly matched lawn chairs (from yard sales; no 2 alike). The bottle eventually was emptied and we lamented its passing, but the next evening as I perchance opened the cupboard, there was a new bottle. Frank had mysteriously found a place to replenish the elixir, but of course he never explained his action. I must admit that as we sat and sipped that scotch the topic of conversation was often virology. Afterward, my son Rick sent Frank a copy of his video of the trip, so there was much email traffic back-and-forth reliving each adventure. Life at its fullest.

Does all this capture Frank Fenner, the man, the friend who has died? If not, then words cannot serve what memories can. Whenever we think of the rise of virology from its roots in pathology and the infectious disease sciences, to its flowering in the first laboratory experiments in viral biology and pathogenesis, and to the beginnings of molecular virology, the name Frank Fenner will be remembered. Whenever we who knew him as a friend think of the emergence of virology as a distinct discipline,

at the time of the first International Congresses of Virology (1968 forward), his role will be remembered with great fondness. Whenever we extend these memories to the more personal aspects of life, Frank's quiet, unassuming character, a character with a steel spine and great insight and understanding of people, will be recalled, again with fondness mixed with great respect. He will never be forgotten.

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Eugène-Ernest Hillemacher (1818–1887) *Edward Jenner Vaccinating a Boy* (1884) Oil on canvas (73.1 cm × 92.7 cm). Copyright Wellcome Library, London

The Fragrance of the Heifer's Breath

Polyxeni Potter

Smallpox was "the most terrible of all the ministers of death," wrote English historian Thomas Macaulay. In the 17th century, the "small pox was always present," unlike the plague, which visited on occasion. Before this time, the disease did not pose a serious threat to the English or other nations in Europe perhaps because, as scant available records suggest, less virulent forms of the disease were endemic. In his account of Queen Mary's death (1694), Macaulay spoke of smallpox as indiscriminate and pernicious, attacking royalty and the poor alike, "turning the babe into a changeling at which the mother shuddered and making the eyes and cheeks of the betrothed maiden objects of horror to the lover."

Early in the century, the disease started appearing in English poetry, and in 1616, the year of Shakespeare's death, Ben Johnson published "An Epigram to the Smallpox": "Envious and foule Disease, could there not be / One beauty in an Age, and free from thee?" These writings reflected growing awareness of smallpox as debilitating and deadly. By the end of the century, it had overtaken not only the plague but leprosy and the great pox, syphilis, and was spreading quickly in communities, disrupting the social fabric and causing epidemics similar to those that would sweep the globe in the 18th and 19th centuries. The general belief was that smallpox was carried from the sick on clothes and bedding, though the primary source of infection was actually particles of moisture in the patient's breath. Infection traveled hundreds of yards by air, and the virus entered the body through the nose and mouth rather than cuts in the skin.

Malodorous and disfiguring, smallpox held none of the mystique of tuberculosis, whose wasting febrile victims would later inspire romantic literature, music, and art. On the contrary, it struck fear in the hearts of poets, from Andrew Marvel to John Donne. Those who tried their hand at elegies struggled to align this "loathsome and unlovely" condition with internal beauty. A tribute by a young John Dryden to his friend Lord Hastings, who died the day before he was to marry, illustrates the pitfalls: "Blisters with pride swell'd; which th'row's flesh did sprout / Like Rose-buds, stuck i'th' Lily-skin about. / Each little Pimple had a Tear in it, / to wail the fault its rising did commit: / Who, Rebel-like, with their own Lord at strife, / Thus made an Insurrection 'gainst his Life."

Dryden's attempt in his first published poem to liken Hastings' pustules to rosebuds and later to gems and stars failed to transform smallpox to anything palatable. Dryden was not alone in confronting the reality of pathologic symptoms in a poem, but this disease, an everyday event, defied metaphorical interpretation and therefore transformative treatment. Moreover, it was not viewed as divine punishment for "Capital offense, / Some high, high Treason." It remained instead, a "Fierce disease, which knows not how to spare / The young, the great, the knowing, or the Fair."

Records from Glasgow show that in the immediate prevaccination era (1783–1800), 50% of children died before age 10, and of those deaths, 40% were due to smallpox, the leading cause of blindness in Europe. Those who survived were often badly blemished, "Beauty's Enemy" lingering "in many a pityed face / Those hatefull pits and furrowes of its trace."

The roots of smallpox in antiquity have been argued, as have efforts to control it. The practice of variolation—inoculation with a small amount of material from a pustule or scab of a smallpox patient—had long been known in Asia and was introduced to Europe and North America in the early 1700s. But it was not widely practiced because of the risk for disease or death to the inoculated person and the risk for creating new outbreaks.

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Then Edward Jenner (1749–1823) came along. An alert physician, he observed that some in his community, mostly farmers and milkmaids frequently exposed to cowpox, did not come down with smallpox. Bucking anecdotal evidence and standard variolation, he went out on a limb. "The first experiment (14 May 1796) was made upon a lad of the name of Phipps, in whose arm a little Vaccine Virus was inserted taken from the hand of a young woman who had been accidentally infected by a cow…. On his being inoculated some months afterwards, it proved that he was secure…. As soon as I could again furnish myself with Virus from the Cow, I made an arrangement for a series of inoculations."

Jenner wrote his observations in his *Inquiry into the Causes and Effects of the Variolae Vaccinae, A Disease Discovered in Some of the Western Counties of England, Particularly Gloucestershire, and Known by the Name of the Cow Pox*. He submitted this small but potent study for publication in the Philosophical Transactions of the Royal Society of which he was a member recognized for his contributions to the field of natural history. The manuscript was rejected, so he published it himself.

An early practitioner of One Medicine, Jenner referred to the cowpox material as "vaccine," from the Latin *vacca* (cow), acknowledging the essential link between human and animal health. Eventually, the procedure of injecting the vaccine was generally referred to as vaccination. Louis Pasteur later insisted that all inoculations designed to protect against disease be called vaccinations in honor of Jenner and named his own discovery "rabies vaccine," though it had no connection with cows.

Edward Jenner Vaccinating a Boy, on this month's cover, commemorates vaccination against smallpox by the country doctor from Berkeley, England, who first demonstrated its effectiveness. Jenner, relying on epidemiologic observations, transformed haphazard efforts against smallpox into a public health approach to disease control and laid the foundation for eradication of this disease and the prevention of many others. This historic moment was captured by Eugène-Ernest Hillemacher, a French painter who worked in the tradition of William Bouguereau and Jean-Léon Gérôme—academic painters known for their affection toward historical subjects. A student of Léon Cogniet, Hillemacher exhibited in the Salon and became Chevalier de la Légion d'Honneur.

The artist's rendition of Jenner's moment in history carefully recorded it for posterity. This traditional bucolic setting shows a family in their modest country home. The back of a cow sharing the premises is visible behind the crib. The good doctor is fully engaged, comforting the young patient who is about to feel a prick. The parents lean forward trustingly. Ample light and bright colors denote optimism. This family's future is safe from pestilential illness. But Jenner here is not tending this child alone but all children. He is pioneering public health's crowning achievement, childhood immunization.

While the specter of smallpox did not translate into lyrical verse, the prospect of its relief resonated with poets and artists. Samuel Taylor Coleridge, whose son died after being variolated, was inspired by Jenner's bold discovery and wrote to him. Eliciting the genius of John Milton, he rated the discovery "capable in the highest degree of being poetically treated," by the bard's very definition of poetry as "simple, sensuous, and impassioned." Coleridge did not follow up, but other Romantic poets did. In 1804, Robert Bloomfield wrote in "Good Tidings or News from the Farm" about the "fragrance of the heifer's breath."

In the 180 years between Jenner's earthshaking experiment and the fulfillment of his vision that "the annihilation of the Small Pox… must be the final result of this practice," he was often berated and ridiculed, particularly for injecting animal material into humans, thus diminishing the distance between them and unclean or sick beasts. We have since learned enough about our close kinship with the animal kingdom, and the dangers involved, to look back at the origins of "vaccine" with affection. Unlike humans whose breath spread smallpox to those who "breath'd the tainted air," Jenner's heifer lived in the house with the farm family, sharing nothing but her fragrant breath.

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Article Title

H275Y Mutant Pandemic (H1N1) 2009 Virus in Immunocompromised Patients

CME Questions

1. You are seeing a 50-year-old man being treated for acute myeloid leukemia. He complains of 3 days of fever, chills, fatigue, and wet cough. You suspect influenza, which has been more prevalent in your area over the last month, but you are concerned regarding possible antiviral resistance in this high-risk patient. Which of the following statements regarding the clinical characteristics of patients in the current study who have resistant pandemic (H1N1) 2009 is most accurate?

A. Only adults, not children, had pandemic (H1N1) 2009 with the H275Y mutation
B. Most cases of resistant pandemic (H1N1) 2009 were generated from contact with other infected patients
C. Most cases of resistant pandemic (H1N1) 2009 were diagnosed more than 5 days after the onset of symptoms
D. Lymphopenia was present in nearly half of patients with the H275Y mutation

2. A rapid polymerase chain reaction (PCR) test is positive for the patient in question number 1. Which of the following statements regarding outcomes of patients with the H275Y mutation in the current study is most accurate?

A. No patients died
B. The H275Y mutation was associated with higher levels of virulence
C. Viral shedding was prolonged, even after clinical recovery
D. No patient receiving only oseltamivir experienced clinical recovery

3. What was the rate of mutation development among all infected patients in the current study?

A. 4%
B. 16%
C. 28%
D. 50%

4. Which of the following statements regarding the virology of cases in the current study is most accurate?

A. Pandemic (H1N1) 2009 was detected in all tissues in the patient with the most severe illness
B. There was generally no discrepancy between genotyping from nasal wash and bronchoalveolar lavage samples
C. The combination of oseltamivir plus rimantadine prevented the development of the H275Y mutation
D. The initial viral load had no effect on the emergence of resistance

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Mumps Complications and Effects of Mumps Vaccination, England and Wales, 2002–2006

CME Questions

1. A 3-year-old child contracts mumps and is hospitalized. Which of the following is of most concern as a long-term complication?

A. Aseptic meningitis
B. Sensorineural deafness
C. Pancreatitis
D. Oophoritis

2. Which of the following best describes the notification rate of mumps infection in England and Wales between 2003 and 2004?

A. Similar attack rates
B. Increased by 2 times
C. Increased by 3 times
D. Increased by 4 times

3. Which of the following mumps complications was associated with the highest hospitalization rate in the mumps epidemic of 2004–2005 in England and Wales?

A. Pancreatitis
B. Orchitis
C. Meningitis
D. Encephalitis

4. A single dose of MMR vaccination compared with no MMR vaccination is likely to have which of the following impact on the risk for hospitalization from mumps infection?

A. Not reduced
B. Reduced by 15%
C. Reduced by 25%
D. Reduced by 50%

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Lessons Learned about Pneumonic Plague Diagnosis, Democratic Republic of the Congo

Intravenous Artesunate for Severe Malaria in Travelers, Europe

Transstadial Transmission of *Francisella tularensis holarctica* in Mosquitoes, Sweden

Severe Imported *Plasmodium falciparum* Malaria, France, 1996–2003

Plasmodium knowlesi Malaria in Children

Experimental Oral Transmission of Atypical Scrapie to Sheep

Travel-related Dengue Virus Infection, the Netherlands, 2006–2007

Molecular Epidemiology of Oropouche Virus, Brazil

Babesiosis, Lower Hudson Valley Region, New York

Clinical Features of *Streptococcus suis* Serotype 2 Infections, Thailand

Experimental Infection of *Amblyomma aureolatum* Ticks with *Rickettsia rickettsii*

Evolution of New Genotype of West Nile Virus in North America

Evidence of Tungiasis in Pre-Hispanic America

Linguatula serrata in the Human Eye

Spotted Fever Group Rickettsiae in Ticks, Germany

Rickettsia rickettsii Transmission by *Amblyomma americanum* Ticks

Increase in Hospitalizations for Dengue, United States

Detection and Phylogenetic Characterization of Human Hepatitis E Virus Strains, Czech Republic

Genetic Characterization of West Nile Virus, Lineage 2, Greece, 2010

Tick-Borne Relapsing Fever Borreliosis, Rural Senegal

Tick-Borne Encephalitis, Kyrgyz Republic

Dengue Virus Serotype 3 Imported from Yemen to Italy

Complete list of articles in the May issue at
http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 1–4, 2011
Annual Scientific Meeting of The Society for Healthcare Epidemiology of America
(SHEA) 2011
Dallas, Texas, USA
http://www.shea2011.com

April 13–16, 2011
The Denver TB Course
Denver, CO, USA
http://www.njhealth.org/TBCourse

June 15–17, 2011
The 61st Annual James Steele Conference on Diseases in Nature Transmissible to Man (DIN)
Omni Bayfront Hotel
Corpus Christi, Texas, USA
http://www.omnihotels.com/Home/FindAHotel/CorpusChristiBayfront.aspx

July 8–10, 2011
International Society for Infectious Diseases Neglected Tropical Diseases Meeting (ISID-NTD)
Boston, MA, USA
http://ntd.isid.org

September 17-20, 2011
51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
McCormick Place Chicago
Chicago, Illinois, USA
www.icaac.org

October 12–15, 2011
The Denver TB Course
Denver, CO, USA
http://www.njhealth.org/TBCourse

November 16–19, 2011
7th World Congress of the World Society for Pediatric Infectious Diseases (WSPID 2011)
Melbourne, Australia
http://www.kenes.com/wspid2011/mailshot/ms3.htm

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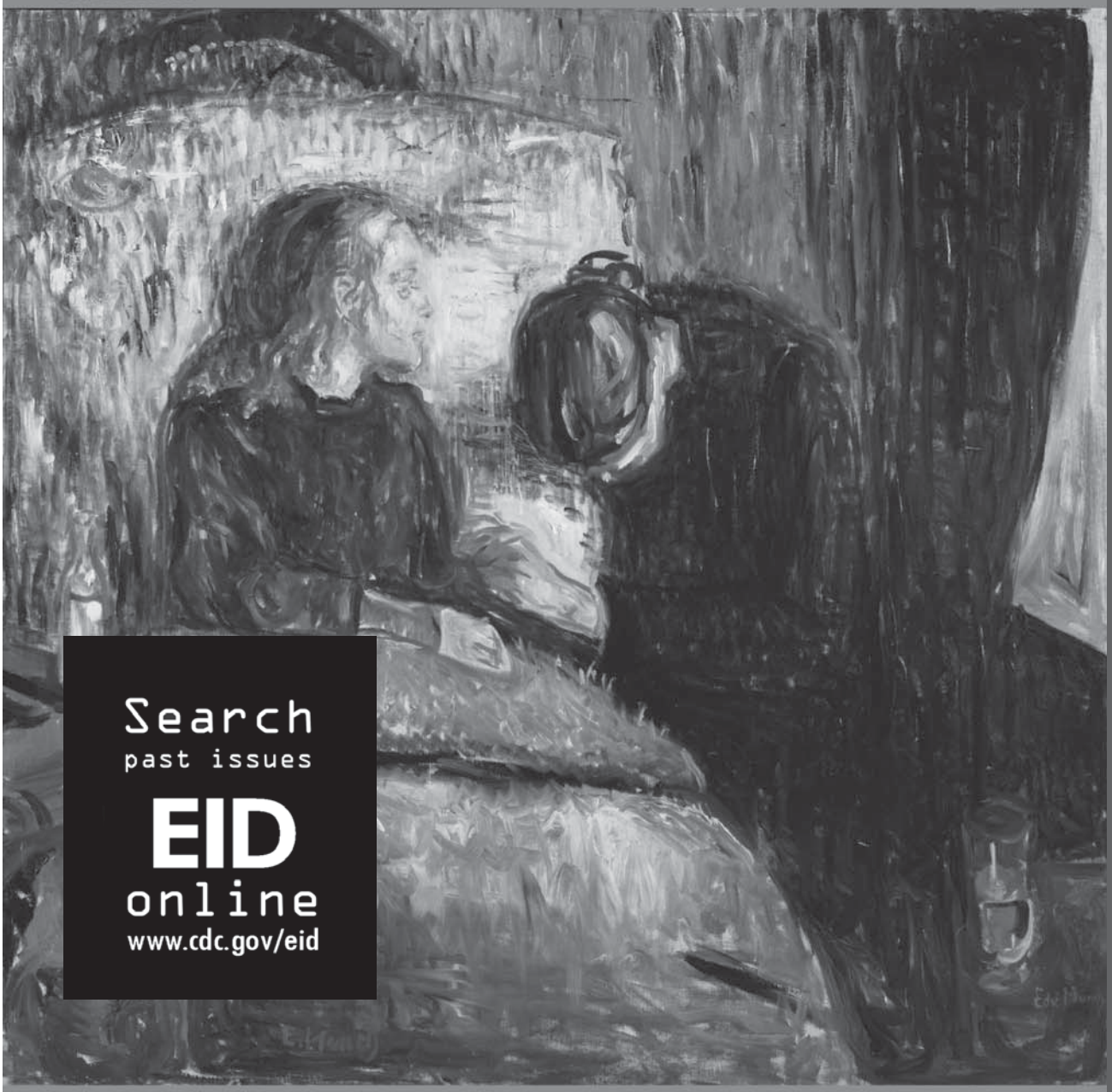
EMERGING INFECTIOUS DISEASES®

March 2011

Tuberculosis



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Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.

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Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.